# Sequence of bovine carbonic anhydrase VI: potential recognition sites for *N*-acetylgalactosaminyltransferase

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Carbonic anhydrases (CAs I–VII) are products of a gene family that encodes seven isoenzymes and several CA-related proteins. We report the cloning and sequencing of the cDNA clones encoding one of these isoenzymes, CA VI, from bovine submaxillary gland. The translated polypeptide consists of 319 amino acids, including a signal peptide (14 amino acids) typical of secreted proteins. The predicted mature protein contains 305 amino acids including a 13-amino-acid C-terminal sequence that is also present in the sheep but absent in human CA VI. The deduced mature bovine protein is 87% and 68% identical to that of sheep and human CA VI, respectively. Active-site residues of the enzyme, as well as the three zinc-binding histidines and the two cysteines involved in an intra-chain disulphide bond, are all conserved in the three species. Two potential Asn-glycosylation sites are also conserved, both of which appear to be glycosylated in sheep and bovine CA VI. Two potential peptide recognition sequences are present in bovine CA VI for the glycoprotein hormone: *N*-acetylgalactosaminyltransferase (GalNAc-transferase), which is one of the two transferases required to form GalNAc-4-$SO_4$ in bovine CA VI-linked oligosaccharides. Specifically, these two sequences are Asp-Leu-Lys-Met-Lys-Lys and Ile-Thr-Lys-Arg-Lys-Lys. Comparison of these sequences with sheep and human CA VI sequences indicates that distinct glycoforms of CA VI could exist in submaxillary gland from different species.

## INTRODUCTION

Carbonic anhydrase (CA, EC 4.2.1.1) catalyses the reversible hydration of carbon dioxide. There are seven distinct isoenzymes present in mammalian tissues that differ in their kinetics, in subcellular localization, and in tissue distribution. These isoenzymes are required in a variety of physiological processes that involve pH regulation, $CO_2$ and $HCO_3^-$ transport, ion transport, and water and electrolyte balance. CAs (CAs I–VII) and CA-related proteins share significant sequence similarity and are encoded by the same gene family [1].

The secreted isoenzyme, CA VI, has been found in the saliva and parotid glands of a number of mammalian species [2]. It is a glycoprotein with an apparent subunit molecular mass of about 45000 Da, as estimated from SDS/PAGE. Removal of the Asn-linked carbohydrates reduces the molecular mass to 36000 Da [3,4]. The amino acid sequence of sheep CA VI, determined by protein sequencing, consists of 307 amino acids [5]. The amino acid sequence of human CA VI, deduced from the cloned cDNA, contains 308 amino acids including a signal peptide of 17 amino acids [6]. A major difference between human and sheep sequences (72% overall identity) is that 13 amino acids at the C-terminus of sheep CA VI are missing in the human CA VI. The function of this region, which is very hydrophilic, is not known.

Distinct glycoforms of CA VI have been found in bovine submaxillary and parotid glands [7]. More than half of the oligosaccharides (55%) released from submaxillary CA VI contain branches terminating with *N*-acetylgalactosamine-4-$SO_4$ (GalNAc-4-$SO_4$), whereas the parotid CA VI contains no sulphate in its oligosaccharides terminated with GalNAc. These tissue-specific glycoforms of bovine CA VI are due to differential expression of a GalNAc-transferase and a GalNAc-4-sulphotransferase, the two enzymes required to form GalNAc-4-$SO_4$. The glycoprotein hormones lutropin and thyrotropin were the first known substrates of these transferases; as a result, the first enzyme was designated as the glycoprotein hormone: GalNAc-transferase (referred to herein as the GalNAc-transferase) [8,9].

To synthesize sulphated oligosaccharides on the lutropin-related glycoprotein hormones, the GalNAc-transferase requires a specific recognition motif, Pro-Leu-Arg-Ser-Lys-Lys, which is contained within a 22-amino-acid glycopeptide fragment of their $\alpha$ subunit [10]. This fragment has been shown for human chorionic gonadotropin to contain all of the information required for recognition by the GalNAc-transferase [11]. The role of individual amino acids except serine in the motif has been examined by site-directed mutagenesis [10]. In this motif, Lys-Lys is essential, Arg can only be replaced by Lys, Leu plays a modulatory role, and Pro is not essential for recognition. The critical role of the three basic residues is further supported by their presence in the $\alpha$ subunits of the lutropin-related glycoprotein hormones from all vertebrate species examined [12].

To synthesize the sulphated GalNAc on Asn-linked saccharides on CA VI, a similar peptide motif might be necessary. By analogy to the glycoprotein hormone $\alpha$ subunit, candidate sequences in CA VI for recognition by the GalNAc-transferase, Pro-Lys-Arg-Lys-Lys and Pro-Leu-Lys-His-Arg, have been proposed for sheep and human enzymes, respectively [7]. However, these sequences begin with Pro, which is not essential for the recognition by the GalNAc-transferase, and the human sequence does not contain Lys-Lys. In addition, oligosaccharides of sheep and human CA VI have not been shown to contain the sulphated

Abbreviations used: CA, carbonic anhydrase; GalNAc, *N*-acetylgalactosamine.

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The sequence of bovine carbonic anhydrase VI has been deposited in the EMBL database under the accession number X96503.

GalNAc, and the bovine CA VI amino acid sequence is not yet available.

We now report the nucleotide sequence and deduced amino acid sequence for bovine CA VI. This is the first nucleotide sequence reported for any CA isoenzyme in this species. The deduced amino acid sequence has two potential recognition sites for the GalNAc-transferase which differ from the candidate sequences previously proposed for sheep and human CA VI and resemble more closely the recognition motif in the $\alpha$ subunit of the glycoprotein hormones. Comparison of these sites with sheep and human CA VI sequences indicates that distinct glycoforms of CA VI could exist in submaxillary gland from different species.

## EXPERIMENTAL

### Construction of a bovine submaxillary cDNA library

Total RNA was isolated from the submaxillary gland of one bovine using a guanidine isothiocyanate/CsCl density gradient centrifugation procedure [13]. Poly(A) RNA was isolated from total RNA by chromatography on oligo(dT)-cellulose [14]. Both total and poly(A) RNA were analysed by Northern blot analysis with radiolabelled actin as a probe and cell-free translation to ensure the quality of the RNA preparation. The cDNA library was constructed using a $\lambda$ZAP-cDNA synthesis kit (Stratagene). The library was amplified once and stored at 4 °C.

### Screening of the cDNA library

The library was initially screened with a cDNA probe encoding a bovine submaxillary mucin [15]. This work will be described elsewhere. One clone containing a partial mucin sequence and a partial CA VI sequence was found from the initial screening. A fragment from this clone containing both mucin and CA VI sequences was used to screen the library. The library was plated on to LB plates supplemented with 10 mM $MgSO_4$ and 0.2% maltose. Each 150-mm-diam. plate covered 10 to 100000 plaques. Duplicate filter lifts were performed using Immobilon-NC (HATF) membranes (Millipore). These filters were cross-linked in a UV cross-linker (Fisher), and prehybridized at 42 °C for 2–4 h in a solution containing 30% formamide, 6 × SSC, 5 × Denhardt's, 0.5% SDS and 100 $\mu$g/ml fragmented herring sperm DNA. (1 × SSC: 0.15 M NaCl/0.015 M sodium citrate.) These filters were then hybridized at 42 °C overnight in a fresh solution containing the radiolabelled probe at a concentration of greater than $1 \times 10^6$ c.p.m./ml. These filters were washed, and exposed to X-ray film (Kodak). Putative plaques were further verified by secondary screening. Positive clones were isolated and their inserts were subcloned into pBluescript SK- by *in vivo* excision with the helper phage R408 (Stratagene). Plasmids were isolated using the Wizard Mini-Prep kit (Promega).

### DNA sequencing and sequence analysis

The plasmids and their subclones were sequenced using the Sanger dideoxy termination method with the Sequenase kit from Amersham [16]. The primers for pBluescript (T3 and T7) were used as sequencing primers. In addition, internal oligonucleotides were synthesized based on sequences obtained, and used as sequencing primers. Both strands were completely sequenced. The nucleotide sequence and deduced amino acid sequence were used to search DNA and protein databases to find homologous sequences using the BLAST e-mail server at the National Center of Biotechnology Information [17]. The deduced amino acid sequence was aligned to sheep and human CA VI using Clastal V [18]. Secondary structures were predicted by the PHD program [19], and further verified by comparison of the predicted structures with known CA structures in the Protein Data Bank using the SCOP program [20].

## RESULTS AND DISCUSSION

### Identification and sequencing of the cDNA clones for CA VI

In the course of sequencing mucin or mucin-like clones from the bovine submaxillary cDNA library, the deduced amino acid sequence of one region from clone 421 was found to be very similar to the C-terminal half of human and sheep CA VI (70 and 90% identical to the corresponding part of the human and sheep CA VI, respectively). This region was linked to a cDNA fragment encoding part of the bovine submaxillary mucin. How this fusion occurred is not known although the restriction enzymes (*Eco*RI and *Xho*I) used in the library construction were found not to be involved. Rescreening of the same library with the fragment containing the fused regions identified more than 30 clones which were further characterized by Southern blot analysis using the CA VI-specific and the mucin-specific probes. Many of these clones hybridized to the CA VI-specific probe, and none of them hybridized to the mucin-specific probe. Thus, the CA VI fragments were not fused to the mucin fragments in the newly identified clones, and fusion of CA VI and mucin in clone 421 represented a rare event in the library. The presence of multiple CA VI clones in the cDNA library indicates abundance of CA VI mRNA in the submaxillary gland, which agrees with the fact that CA VI is expressed at very high levels in this tissue [4]. Several of the CA VI-specific clones were sequenced, and found to overlap with the CA VI-specific probe and extend further in the 5′ direction. The sequencing strategy and results are summarized in Figure 1.

### Nucleotide sequence and the deduced amino acid sequence of bovine CA VI

Several lines of evidence indicate that the nucleotide sequence in Figure 2 represents the full-length cDNA of bovine CA VI. First, 24 out of 25 residues at the N-terminus of the predicted mature protein match those reported for purified bovine CA VI [2]. The only mismatch is His-2 in the deduced sequence versus Ser-2 from protein sequencing. The reason for this discrepancy is not known, but could be due to allelic differences in different bovine breeds, or an error from protein sequencing (His-2 is also present

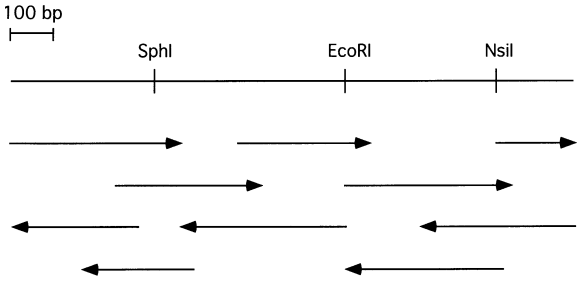


**Figure 1 Sequencing strategy of bovine CA VI**

The restriction enzymes shown above were used to construct subclones for sequencing. Arrows indicate the extent and direction of the sequencing information obtained. Primers (T3 and T7) for the vector, pBluescript SK-, and synthetic oligonucleotides were used as primers for sequencing reactions.

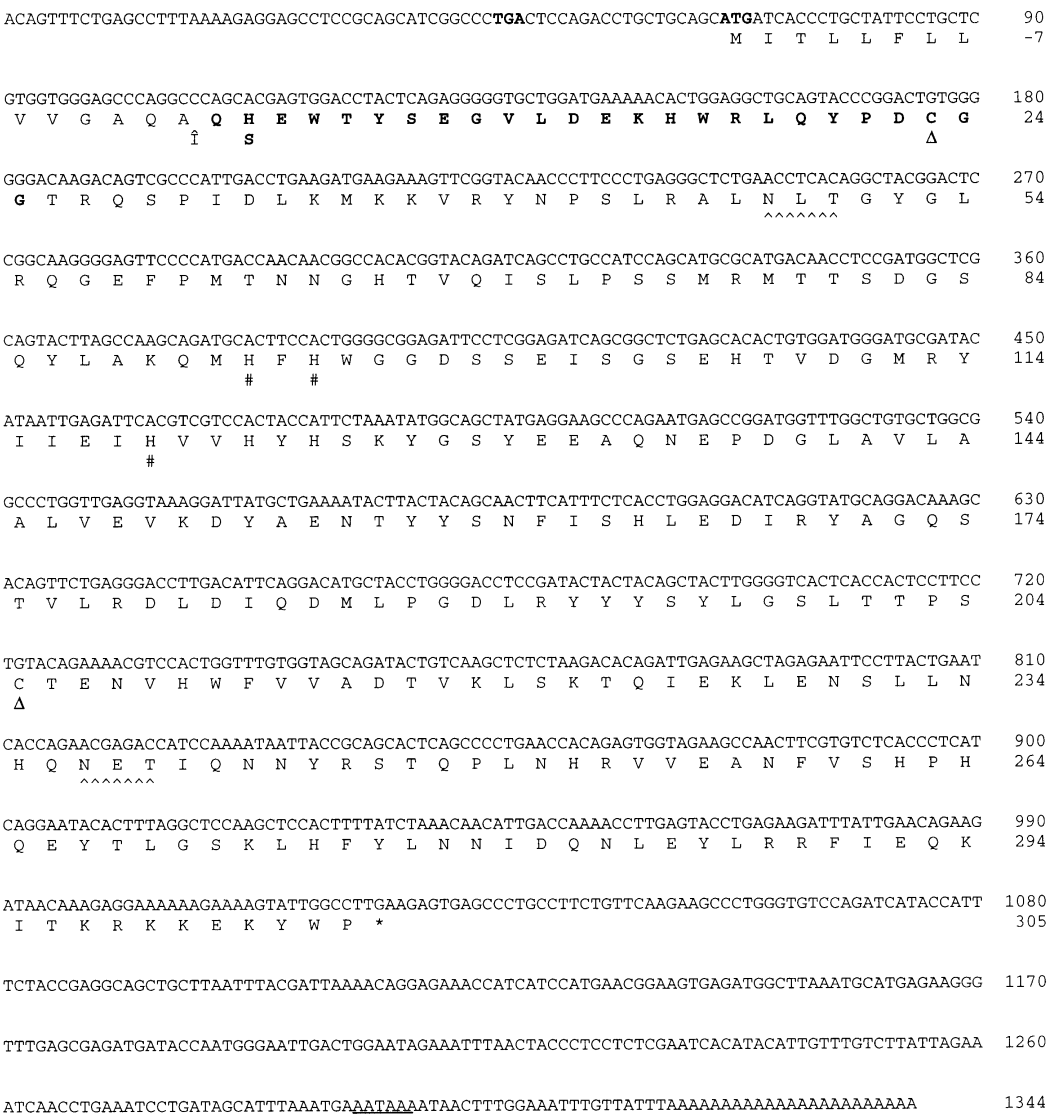


**Figure 2 The cDNA and deduced amino acid sequence of bovine CA VI**

The initiation codon for translation, the in-frame stop codon before that, and the N-terminal sequence determined for the purified enzyme [2] are shown in bold typeface. The only difference is His-2 versus Ser-2 by protein sequencing, as indicated below the deduced sequence. The potential cleavage site for a signal peptide is indicated by Î. The numbering for amino acids starts with the N-terminus of the mature protein. The two Cys residues that form an intra-chain disulphide bond in sheep CA VI are indicated by triangles. The potential N-glycosylation sites are indicated by carets. The three His residues involved in zinc-binding are indicated by #. The putative polyadenylation signal is underlined.

in sheep and human CA VI). Secondly, the deduced amino acid sequence shows a high sequence similarity with sheep and human CA VI, as described below. Thirdly, Northern blot analysis of total RNA from various bovine tissues detected a 1.4 kb mRNA only in submaxillary gland (results not shown), which is close to the length (1344 bp) of the cDNA.

The predicted mature protein has several characteristics that are similar to CA VI isolated from bovine submaxillary gland [7]. The protein consists of 305 amino acids with a calculated molecular mass of 35540 Da which is close to the size (35000 Da) of the deglycosylated protein. The polypeptide contains two potential N-glycosylation sites, both of which appear to be glycosylated in purified CA VI. An additional 14 amino acids are deduced from the cDNA before the start of the predicted mature protein (Figure 2). These residues constitute a hydrophobic sequence that could represent a signal peptide present in most secreted proteins [21].

There are several additional features in the cDNA sequence. The first in-frame ATG in the open reading frame resides within a sequence that is similar to a vertebrate consensus sequence for ribosome binding [22]. The role of this ATG as the initiation codon for translation is further supported by a preceding in-frame termination codon TGA. A polyadenylation consensus signal is present 29 nucleotides upstream from the poly(A) tail [23].

## Sequence comparison of CA VI from bovine, sheep and human

The amino acid sequence of the deduced mature bovine protein is 87% and 68% identical to sheep and human CA VI,


**Figure 3 Comparison of the amino acid sequences of bovine, sheep, and human CA VI and potential recognition sites in CA VI for the GalNAc-transferase**

Alignment was performed using Clustal V [18]. Identical residues are indicated by stars and conservative substitutions by dots. Deletions are indicated by dashes. The $\alpha$ subunit fragment of 22 amino acids (residues 35 to 56) from human chorionic gonadotropin (hCGalpha) that contains all of the information required for recognition by the GalNAc-transferase [11] is included for comparison above the alignment where the recognition site is underlined, as is the potential recognition site in bovine or sheep CA VI. Below the alignment are the secondary structures predicted for bovine CA VI by the PHD program [19], and further verified by comparison of the predicted structures with known CA structures in the Protein Data Bank using the SCOP program [20]. Abbreviations: h, a helix element; s, a $\beta$-strand element.

respectively (Figure 3). Two Cys residues known to form an intra-chain disulphide bond in sheep CA VI are conserved, as are three Asn-Xaa-Thr/Ser triplets, the last two of which are known to be glycosylated in sheep CA VI [5]. Aldred et al. proposed that the first triplet was a potential N-glycosylation site in human CA VI as well [6], which is unlikely because Xaa is a Pro which is known to inhibit N-glycosylation [24,25].

It is noteworthy that like the sheep CA VI, the C-terminus of the bovine sequence extends 13 amino acids beyond the end of the human CA VI. This difference between bovine and human protein sequences was further examined by comparing their nucleotide sequences (Figure 4). In the 5′ untranslated region, the bovine sequence contains 66 nucleotides as compared with four nucleotides reported for the human sequence [6]. In the coding region, deletion or insertion of nucleotides occurs in triplets and coincides with codons (therefore amino acids) with the following single exception. A single deoxythymidine in the bovine sequence is deleted in the human sequence between nucleotides 921 and 922. This deletion causes a change in the reading frame of human CA VI that terminates at the stop codon (nucleotides 929–931), and results in a polypeptide chain lacking the last 13 amino acids present in the C-terminus of bovine and sheep CA VI. This change could be due to species difference, or it could be the result of an artifact in cloning or sequencing of human cDNA. Although further analysis of the human CA VI cDNA or protein is required to distinguish the two possibilities, the latter possibility is more likely because the sequence of human CA VI after nucleotide 809 was determined from the cDNA obtained by the PCR using *Taq* DNA polymerase which is known to produce errors [26].

The deduced amino acid sequence of bovine CA VI is 31% identical to bovine CA II, the only other CA amino acid sequence available from bovine tissues [27]. CA VI contains 45 more amino acids than CA II at its C-terminus. The low identity between CA VI and CA II indicates that CA VI and CA II diverged from an ancestral CA gene at an early stage of evolution. Nevertheless, the important residues are conserved, including the active-site residues and three histidines that bind to zinc.

## Potential recognition sites in bovine CA VI for the GalNAc-transferase

There is no significant similarity between overall amino acid sequences of bovine CA VI and the glycoprotein hormones (such as lutropin and chorionic gonadotropin), both of which are the substrates for the GalNAc-transferase. Thus, it is of interest that two sequences similar to the recognition motif in the glycoprotein

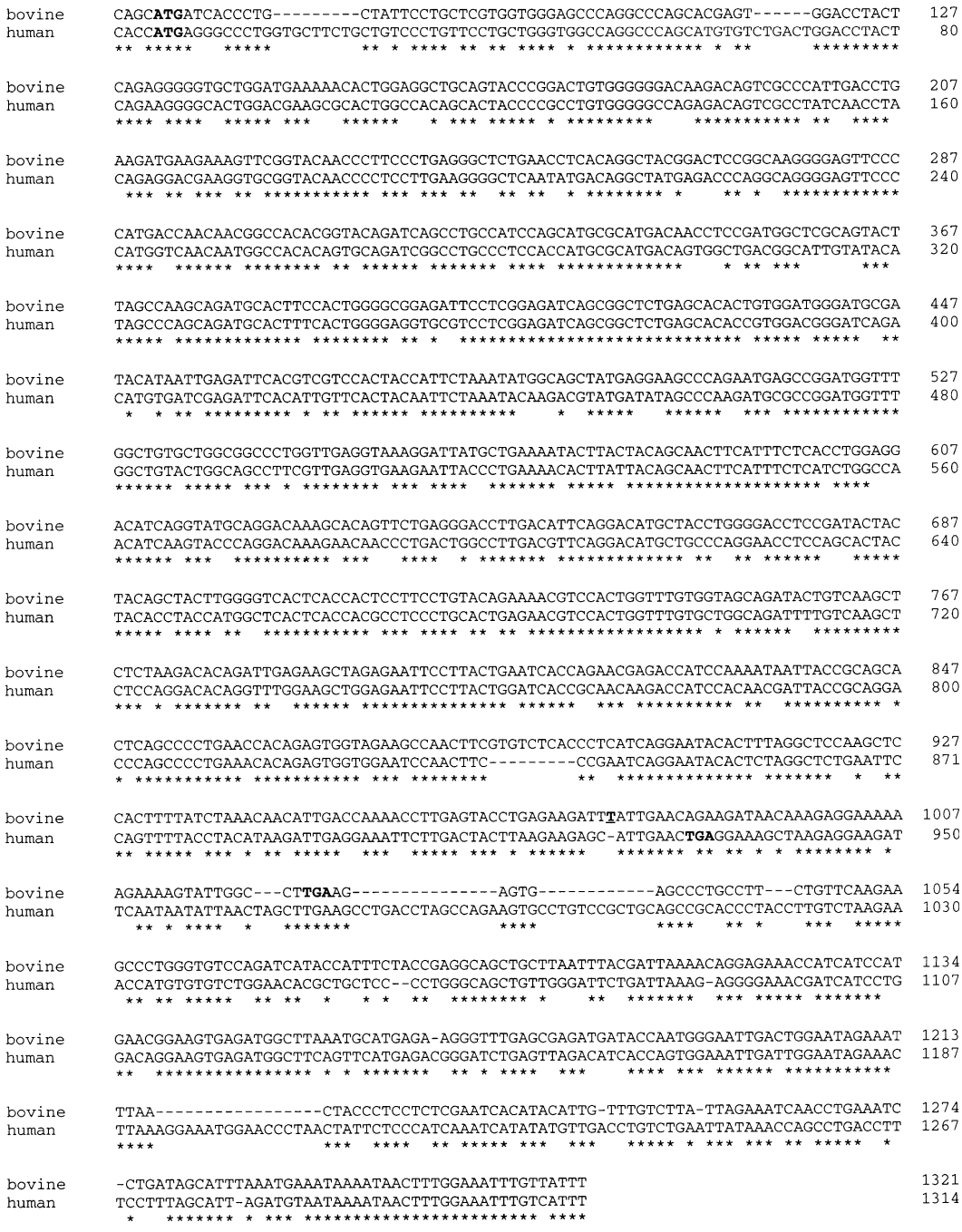


**Figure 4 Comparison of the nucleotide sequences of bovine and human CA VI**

The bovine sequence was aligned to the published human sequence [6] using Clustal V [18]. The first 62 nucleotides of the bovine sequence are not included. Identical nucleotides are indicated by stars. The start and stop codons are shown in bold typeface. The single deoxythymidine present in the bovine sequence and deleted in the human sequence is bold and underlined.

hormones for the GalNAc-transferase are present in bovine CA VI (Figure 3). The first sequence, Asp-Leu-Lys-Met-Lys-Lys, is present 16 residues N-terminal to the first predicted Asn-linked glycosylation site. The second sequence, Ile-Thr-Lys-Arg-Lys-Lys, is present 58 residues C-terminal to the second potential glycosylation site. The first sequence is not conserved in sheep and human CA VI where several substitutions occupy critical positions (Lys residues). The second sequence is conserved in sheep CA VI, but absent in the human sequence. In the primary structure, this sequence seems far away from the glycosylated Asn as compared with the first sequence, but their spatial relationship in the folded protein structure could be much closer. If these two sequences are indeed involved in recognition by the GalNAc-transferase, oligosaccharides linked to both Asn residues of bovine CA VI could contain GalNAc-4-$SO_4$. In comparison, sheep CA VI may contain the sulphated GalNAc only in oligosaccharides linked to the second Asn residue, and human CA VI may not have any sulphated oligosaccharides.

The bovine CA VI sequences containing the potential recognition motifs are further compared with the 22-amino-acid fragment (Figure 3) of the $\alpha$ subunit of human chorionic gonadotropin that contains all of the information required by the GalNAc-transferase [11]. Two more amino acids, Arg and Pro, are conserved between the first bovine sequence and the 22-amino-acid fragment. No further similarity is present between the second bovine sequence and the 22-amino-acid fragment. In addition, the basic amino acids in the two potential recognition motifs in bovine CA VI are not contained within the helical structures predicted by the PHD and SCOP programs [20,21], as are the basic amino acids in the motif of Pro-Leu-Arg-Ser-Lys-Lys from human chorionic gonadotropin determined by X-ray crystallography [28,29]. There are other proteins that do not have any apparent helical structure within the recognition determinant, and human chorionic gonadotropin $\beta$ subunit, a good *in vitro* substrate for the GalNAc-transferase, is such an example [10]. Therefore, it appears that the cluster of basic amino acids required by the GalNAc-transferase can be contained in secondary structures other than helices.

In this report, we provide sequence information on bovine submaxillary CA VI. This is the first bovine nucleotide sequence available for any CA isoenzyme in this species, and the second nucleotide sequence reported for CA VI. The secreted CAs from sheep, bovine and human are similar in their protein structures and tissue-specific expression. Two major differences in the three sequences are the C-terminal region and potential recognition sites for the GalNAc-transferase. The C-terminal region is also part of 45 amino acids that are not present in other CA isoenzymes (260 amino acids or less) [1]. One of the major structural differences between CA VI and other CA isoenzymes is that the former exists as an oligomer of a high molecular mass in the native state [4,30]. It is possible that the C-terminal region of CA VI contributes to association between monomers to form oligomers. If ionic interactions are involved in the formation of oligomers through basic amino acid residues in this region, these interactions could be different between sheep/bovine and human CA VI because the latter lacks the 13 amino acids enriched with basic residues.

It has been shown that the presence of different terminal residues on the Asn-linked oligosaccharides of submaxillary (terminated with GalNAc-4-$SO_4$) and parotid (terminated with GalNAc) CA VI from the same species (bovine) correlates with the complement of the two transferases in these glands [7]. The presence of a different number of potential recognition sites for the GalNAc-transferase in CA VI from different species indicates that the level of GalNAc-4-$SO_4$-terminating oligosaccharides linked to CA VI in submaxillary gland could vary from species to species even when the two transferases are expressed at similar levels. Further structural analysis of the oligosaccharides linked to CA VI from sheep, oxen and humans is required to test this possibility. Based on the effect of the sulphated oligosaccharides on the biological activity of the glycoprotein hormone lutropin [31,32], it is likely that different glycoforms of CA VI from the different species could have different effects on its functions in submaxillary gland.

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