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# mRNA of bovine tissue inhibitor of metalloproteinase: Sequence and expression in bovine ovarian tissue

J. Freudenstein, S. Wagner, R.M. Luck [1], R. Einspanier [2] and K. H. Scheit

Max-Planck-Institut für Biophysikalische Chemie, Abt. Molekulare Biologie,
3400 Göttingen, W.- Germany

[1] Institut für Hormon- und Fortpflanzungsforschung, Grandweg 64,
2000 Hamburg, W.- Germany

[2] Süddeutsche Versuchs- und Forschungsanstalt für Milchwirtschaft Weihenstephan,
Technische Universtät München, Institut für Physiologie,
8050 Freising, W.- Germany

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A cDNA library derived from poly($A^+$)RNA of bovine ovary was screened with a PCR fragment comprising the coding region of human tissue inhibitor of metalloproteinase inhibitor (TIMP). From a number of positive clones, pBGR19, containing a 747 bp insert, was identified and sequenced. The derived amino acid sequence represents that of the precursor of bovine TIMP. Northern analysis reveals a TIMP specific mRNA of 800 bp. Southern analysis indicates that one gene appears to specifiy bovine TIMP. TIMP mRNA is only weakly expressed in follicular granulosa- and theca cells, whereas luteinization of the follicle is associated with an increase of expression. Expression varies with the stage of the luteal phase; it was highest in stages I and III, but low in stages II and IV of the oestrous cycle. 

Ovulation appears to be facilitated by a degradation of the extracellular matrix at the follicular apex which in part results from the action of the metalloproteinase collagenase (1). Biochemical studies demonstrating an increase in collagenase activity during the preovulatory period support this concept (2). The preovulatory increase in collagenolytic activity is stimulated by the LH surge (2, 3); the mechanisms controlling collagenase function in ovulation are, however, unknown. In other tissues, collagenase activity is regulated by several classes of metalloproteinase inhibitors such as $\alpha_2$-macroglobulin and tissue inhibitors of metalloproteinases (TIMP). The latter class of inhibitors possesses a range of molecular weights of 25-30 kDa. Curry et al. (4) identified a metalloproteinase inhibitor activity in human ovarian follicular fluid of $M_r$ 28 kDa very likely to represent human TIMP. By cDNA cloning we recently characterized human TIMP mRNA in granulosa cells of follicles from hormonally

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stimulated female individuals. Northern analysis revealed that this mRNA species represents approximately 2% of the total mRNA of these cells (5). To facilitate an experimental approach towards understanding of regulatory mechanisms controlling the expression of the TIMP gene in granulosa cells we intended to work with an animal model. In this communication we describe the characterization of bovine TIMP mRNA by cDNA cloning as well as a study of the expression of this mRNA in various ovarian cells and tissues.

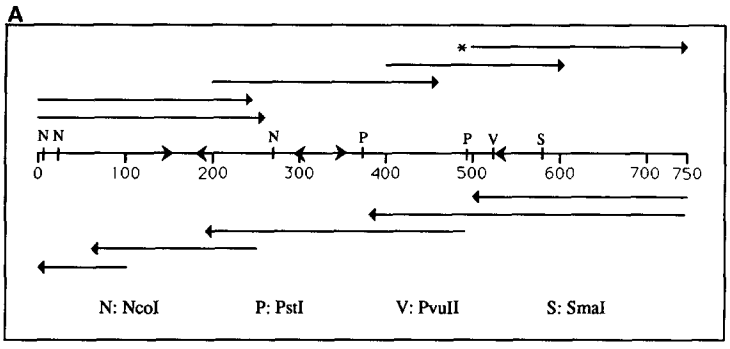
## Material and Methods

Granulosa cells were obtained from large, healthy, antral bovine follicles as previously described (6). Bovine corpora lutea were selected according to the morphological characteristics described by Ireland et al. (7). Theca was taken from follicles used to prepare granulosa cells. Adherent granulosa cells were removed and the tissue was employed for extraction of total RNA. Part of the theca tissue was dispersed into cells enzymatically according to Schultze et al. (8). Total RNA and poly(A+)RNA were prepared as reported (9, 10). cDNA synthesis from bovine granulosa cell poly(A+)RNA followed a modified protocol of Gubler and Hoffmann (11) using a commercial cDNA synthesis kit (BRL). The procedure of Villa-Komaroff et al. (12) was used for the construction of recombinant pBR322. Transformation of *Escherichia coli* RR1 was performed as published (13). The obtained bovine granulosa cell cDNA-library comprised 3 x $10^4$ independent clones with a cDNA insert yield of 60%. The cDNA insert size was in a range of 300-1500 bp. Recombinants (3 x $10^4$) were screened on replica nitrocellulose filters (Millipore HATF) with a [$^{32}$P]-labeled PCR fragment comprising the complete coding region of pHGR122 (5) as hybridization probe. Prehybridization and hybridization were performed according to Hanahan and Meselson (14) at 60°C. Only one positive clone pBGR1 with a cDNA insert of 242 bp could be detected. The *Pst* I fragment of pBGR1 was subcloned into M13mp19 (RF). DNA sequencing was carried out by the dideoxy chain-termination method of Sanger et al. (15). Screening of a commercial λgt10 library derived from bovine ovary (Clontech) employing the hybridization probe as described above and following published procedures (16) furnished a number of positive clones. The *EcoR* I insert of clone pBGR19 comprising ~ 750 bp was subcloned into pUC18 and the sequence was determined by double strand sequencing. For Northern analysis RNA containing 1 M glyoxal was electrophoresed in a 1.5% agarose gel and transferred to nitrocellulose membranes (BAS 85, Schleicher und Schüll). Hybridization of RNA blots strictly followed the protocol of Khandjian (17). Bovine genomic DNA was prepared from blood anticoagulated with EDTA. For Southern blot analysis DNA was digested with restriction enzymes, electrophoresed in 0.7% agarose and transferred to nitrocellulose membranes (BAS 85, Schleicher und Schüll) essentially as described (18). Hybridization was perfomed at 60°C under stringent conditions following the protocol of Church and Gilbert (19). DNA probes were [$^{32}$P]-labeled by random priming to a specific activity of 3 x $10^9$ cpm/μg employing a commercial kit (Amersham).
DNA and protein sequence analysis as well as sequence comparisons were performed using the computer program of the University of Wisconsin Genetics Computer group (20).

## Results and Discussion

A cDNA library derived from bovine granulosa cells was screened by colony hybridization using a PCR fragment of pHGR122 (5) comprising the coding region of the mRNA for human TIMP. Only one clone, pBGR1, with a cDNA insert of 242 bp was detected. The sequence of this insert contained part of the coding as well as the complete 3′- untranslated region. The cDNA insert of pBGR1 possessed an open reading frame from which 46 amino acid residues of the C-terminal position of the

A




B

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1   CGCACCATGGCCCCCTTTGCACCCATGGCCTCTGGCATCCTGTTGCTGCTGTGGCTCACA     60
          M  A  P  F  A  P  M  A  S  G  I  L  L  L  L  W  L  T
        -23       -20            -15            -10

61  GCCCCCAGCCGAGCCTGTACCTGCGTCCCACCCCACCCACAGACGGCCTTCTGCAACTCC    120
    A  P  S  R  A  C  T  C  V  P  P  H  P  Q  T  A  F  C  N  S
 -5           -1 +1            5              10             15

121 GATGTCGTCATCAGGGCCAAGTTCGTGGGGACCGCAGAAGTCAATGAAACTGCCTTATAC    180
    D  V  V  I  R  A  K  F  V  G  T  A  E  V  N  E  T  A  L  Y
                20             25             *******        35

181 CAGCGTTATGAGATCAAGATGACTAAGATGTTCAAAGGGTTCAGTGCCTTGAGGGATGCC    240
    Q  R  Y  E  I  K  M  T  K  M  F  K  G  F  S  A  L  R  D  A
                40             45             50             55

241 CCTGACATCCGGTTCATCTACACCCCTGCCATGGAGAGCGTCTGCGGATACTTCCACAGG    300
    P  D  I  R  F  I  Y  T  P  A  M  E  S  V  C  G  Y  F  H  R
                60             65             70             75

301 TCCCAGAACCGCAGTGAGGAGTTTCTCATAGCTGGACAATTGTCGAATGGGCACCTGCAC    360
    S  Q  N  R  S  E  E  F  L  I  A  G  Q  L  S  N  G  H  L  H
          *******              85             90             95

361 ATCACCACCTGCAGTTTTGTGGCTCCCTGGAACAGCATGAGTTCTGCTCAGCGCCGGGGA    420
    I  T  T  C  S  F  V  A  P  W  N  S  M  S  S  A  Q  R  R  G
             100            105            110            115

421 TTCACCAAGACCTATGCTGCTGGTTGTGAGGAATGCACAGTGTTTCCCTGCTCATCCATC    480
    F  T  K  T  Y  A  A  G  C  E  E  C  T  V  F  P  C  S  S  I
             120            125            130            135

481 CCCTGCAAACTGCAGAGCGACACTCACTGCTTGTGGACGGACCAGCTGCTCACAGGCTCT    540
    P  C  K  L  Q  S  D  T  H  C  L  W  T  D  Q  L  L  T  G  S
             140            145            150            155

541 GACAAGGGGTTCCAGAGCCGCCACCTGGCCTGCCTGCCCCGGGAGCCAGGGCTGTGCACC    600
    D  K  G  F  Q  S  R  H  L  A  C  L  P  R  E  P  G  L  C  T
             160            165            170            175

601 TGGCAGTCCTTGCGGGCCCAGATGGCCTGAGTCCTGCCCCCAGCAGAAGCTGAAGCCCGC    660
    W  Q  S  L  R  A  Q  M  A  *
             180         184

661 ACAGTGTTCGCCCCTGTTTCCACTCCCACCTTTTTTTCTTCAAGAGGATGAAATAAAGAA    720
721 CTACCACCCAGCAAAAAAAAAAAAAAA                                     747
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Fig. 1. cDNA sequence of pBGR19. A: Sequencing strategy and restriction map of pBGR19. The arrow indicated by an asterisk represents the sequence of the cDNA insert of pBGR1. The arrow heads within the map give the position as well as the 5'-3'orientation of the synthetic oligonucleotides employed in double strand sequencing. B: Nucleotide sequence of pBGR19 and deduced amino acid sequence of the precursor of collagenase inhibitor. The amino acid sequence numbering for the mature protein starts with +1. The polyadenylation signal is underlined. Glycosylation sites are marked by asterisks.

bovine TIMP could be deduced due to homology with the corresponding human TIMP (data not shown). Screening a commercial λgt10 library of bovine ovarian tissue as described above furnished a number of positive clones. Clone pBGR19, possessing a cDNA insert of 747 bp, was selected for sequencing by subcloning the *EcoR* I fragment into pUC18. The strategy of double strand sequencing as well as the nucleotide sequence are shown in Fig.1. The nucleotide sequence of the insert of pBGR19 contained an open reading frame (nucleotides 7 to 630) from which the complete amino acid sequence of the precursor of the bovine TIMP could be deduced (Fig.1 B). The translational start ATG is part of a typical eukaryotic consensus sequences for mRNAs GCACCATGG (21, 22, 23). The N-terminal amino acid sequence, 45 amino acid residues for bovine TIMP has been reported (24). Comparison with the deduced amino acid sequence reveals that the N-terminal sequence fragment is incorrect in five positions. The hitherto known amino acid sequences for TIMP precursor molecules of human (25), rabbit (26), mouse (27) and bovine origin are aligned in Fig.2. Sequence comparison for the signal sequences

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     1                                                        50
bov   MAPFAPMAS GILLLLWLTA PSRACTCVPP HPQTAFCNSD VVIRAKFVGT
hum   MAPFEPLAS GILLLLWLIA PSRACTCVPP HPQTAFCNSD LVIRAKFVGT
rab   MAPLAALAS SMLLLLWLVA PSRACTCVPP HPQTAFCNSD LVIRAKFVGA
mus  MMAPFASLAS GILLLLSLIA SSKACSCAPP HPQTAFCNSD LVIRAKFMGS
        Δ             +    Δ   * * ΔΔ #Δ    *

     51                                                      100
bov  AEVNETALYQ RYEIKMTKMF KGFSALRDAP DIRFIYTPAM ESVCGYFHRS
hum  PEVNOTTLYQ RYEIKMTKMY KGFQALGDAA DIRFVYTPAM ESVCGYFHRS
rab  PEVNHTTLYQ RYEIKTTKMF KGFDALGHAT DIRFVYTPAM ESVCGYSHKS
mus  PEINETTLYQ RYKIKMTKML KGFKAVGNAA DIRYAYTPVM ESLCGYAHKS
     Δ                                    Δ      *   #

     101                                                     150
bov  QNRSEEFLIA GQLSNGHLHI TTCSFVAPWN SMSSAQRRGF TKTYAAGCEE
hum  HNRSEEFLIA GKLQDGLLHI TTCSFVAPWN SLSLAQRRGF TKTYTVGCEE
rab  QNRSEEFLIA GQLRNGLLHI TTCSFVVPWN SLSFSQRSGF TKTYAAGCDM
mus  QNRSEEFLIT GRLRNGNLHI SACSFLVPWR TLSPAQQRAF SKTYSAGCGV
                        #    *    Δ+                    *

     151                                                     200
bov  CTVFPCSSIP CKLQSDTHCL WTDQLLTGSD KGFQSRHLAC LPREPGLCTW
hum  CTVFPCLSIP CKLQSGTHCL WTDQLLQGSE KGFQSRHLAC LPREPGLCTW
rab  CTVFACASIP CHLESDTHCL WTDSSL.GSD KGFQSRHLAC LPQEPGLCAW
mus  CTVFPCLSIP CKLESDTHCL WTDQVLVGSE DY.QSRHFAC LPRNPGLCTW
     *   Δ*   Δ *      #*  +                #  *  Δ  Δ  * +

     201
bov  QSLRAQMA
hum  QSLRSQIA
rab  ESLRPRKD
mus  RSLGAR

*: Cysteine      100 %          +: Tryptophan      94 %
#: Histidine      83 %          Δ: Proline         91 %
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Fig. 2.Comparison of amino acid sequences of the precursors of TIMP from bovine, human, rabbit and mouse. Glycosylation signals are underlined; the positions of cysteine, proline, histidine and tryptophan residues are marked.

yields homologies in the range of 64-88%. The extensive sequence homology of the mature polypeptides comprises the full conservation of sequence positions for the 12 cysteine residues as well as the two N-glycosylation sites per molecule, and a 91% homology with regard to proline residues. Furthermore, it is interesting that the cysteine residues, except for cysteine-145, are positioned in regions of nearly identical sequence segments. The high degree of homology for the above TIMP species is also observed at the DNA sequence level. The homology for the coding regions ranges from 75-88% that for the 3´-untranslated sequences from 64-84%. Northern blot analysis of total RNA from bovine granulosa cells employing the cDNA insert of pBGR19 as hybridization probe identified a single mRNA species of ~ 800 bp (Fig. 3). Bovine genomic DNA was analyzed by Southern hybridization. After digestion with *BamH* I, *EcoR* I, *Hind* III and *Kpn* I, enzymes known not to cut within the cDNA insert of pBGR19, hybridization yielded single radiolabeled bands with each digest (Fig. 4). The relative small size of the fragment detected in the *EcoR* I digest

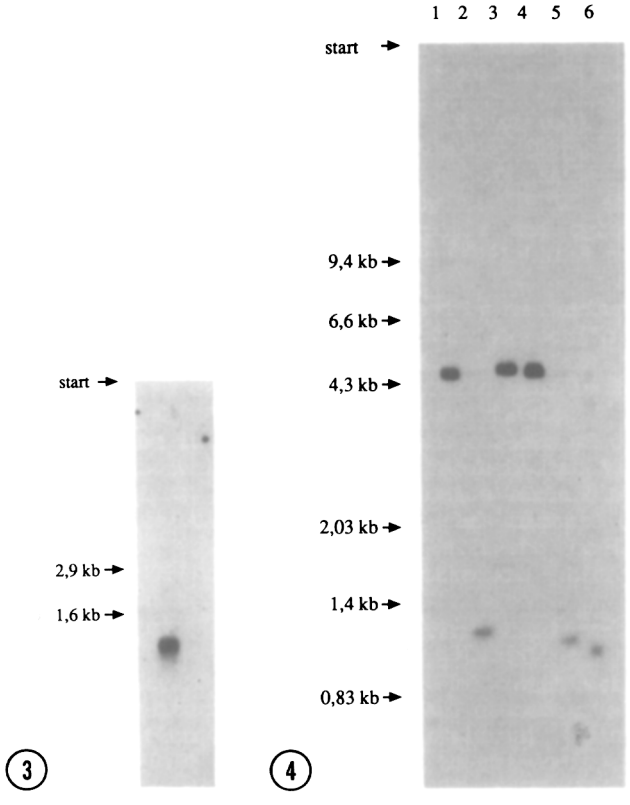


Fig. 3. Northern analysis of poly(A⁺)RNA from bovine granulosa cells. The insert of cDNA clone pBGR19 was employed for hybridization (sp.act. 4 x $10^8$ cpm/µg); 2 µg of poly(A⁺)RNA were subjected to analysis.

Fig. 4. Southern blot analysis of the gene for bovine TIMP. Bovine genomic DNA was digested with *Bam* HI (lane 1), *Eco* RI (lane 2), *Hind* III (lane 3), *Kpn* (lane 4), *Pvu* II (lane 5) and *Sma* I (lane 6). The latter two enzymes are known to cut within the coding region. The insert of cDNA clone pBGR19 was employed as hybridization probe (sp. act. 3 x $10^9$ cpm/µg). Hybridization was performed under stringent conditions.

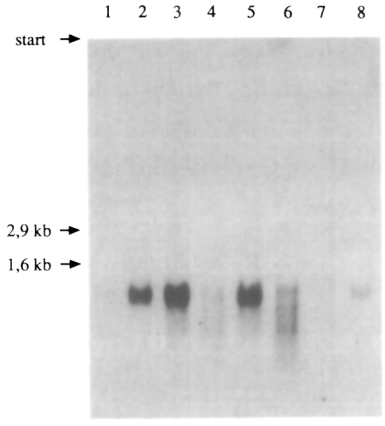


Fig. 5.Northern analysis of total RNA from bovine ovarian tissues. The corpora lutea were selected according to morphological characteristics as described (Ireland et al., 1980). Approximate stages of oestrous cycle were as follows: I, days 1-4; II, days 5-10; III, days 11-17; IV, days 18-20. Lane 1, noncultured granulosa cells; lane 2, granulosa cells cultured in serum-free medium; lane 3, corpus luteum stage I; lane 4, corpus luteum stage II; lane 5, corpus luteum stage III; lane 6, corpus luteum stage IV; lane 7, theca tissue; lane 8, theca cells obtained by enzymatic dispersion. All lanes contained 10 µg of total RNA extracted form the source indicated. Hybridization was performed with the insert of cDNA clone pBGR19 (sp. act. 4 x $10^8$ cpm/µg).

appears to indicate that *EcoR* I sites within the gene are located at exon/intron regions for which the hybridization probe was not specific. We tentatively infer from this experiment that there is one gene for TIMP per haploid bovine genom. Northern analysis of total RNA extracted from various ovarian tissues as well as cells was employed to assess the expression of bovine TIMP. Hybridization was carried out with the [$^{32}$P]-labeled cDNA insert of pBGR19. The data shown in Fig. 5 indicate that TIMP is only weakly expressed in follicular (uncultured) granulosa cells and theca tissue or theca cells. Luteinisation of the follicle is apparently associated with an increase in expression of the TIMP gene. This conclusion is supported by the strong expression of TIMP in cultured granulosa cells which in parallel were shown to express the oxytocin gene (data not shown). The oxytocin gene is expressed in the corpus luteum but not in the follicle of the cow (28) and granulosa cells in serum-free culture spontaneously develop also other characteristics of the luteal phenotype, including secretion of oxytocin, massively increased secretion of progesteron, diminished secretion of oestradiol as well as diminished expression and secretion of inhibin (29). The expression of TIMP may be an additional marker of the luteal phenotype. There was no simple relationship between the level of TIMP expression and the stage of the luteal phase. Expression was highest in stages I and III, but low in stages II and IV of the oestrous cycle. In an independent experiment (data not shown) gene expression of the TIMP gene in corpus luteum of day 12 (high), day 15 (low) and day 17 (high) was analyzed in the same manner with similar results (5). No gene expression was observed in corpus luteum after day 60 of gravidy (data not shown). The increase in expression of the TIMP gene with luteinisation may be significant in relation to the remodelling of follicular tissues after ovulation. Rupture of the follicle

wall results from the action of proteases, including collagenase, released in response to the preovulatory gonadotrophin surge. Following rupture, the theca and granulosa tissues undergo mixing and remodelling during the development of the luteal structure, a process apparently associated with a build up of extracellular matrix. It is reasonable to speculate that remodelling would be facilitated by the presence of TIMP as one antagonist of proteolytic activity.

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