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PORCINE LUTEAL CELLS EXPRESS MONOCYTE CHEMOATTRACTANT PROTEIN-2 (MCP-2): ANALYSIS BY cDNA CLONING AND NORTHERN ANALYSIS

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Summary: From an expression library in λUniZAP, derived from porcine corpus luteum (CL), a clone λMCP9 was detected by hybridization with a porcine MCP-1 specific probe. A pBluescriptSK-derivative pMCP9 was generated from λMCP9 by *in vivo* excision and was shown to contain an open reading frame (ORF) encoding a protein highly homologous to bovine monocyte chemoattractant protein-2 (MCP-2). Comparison of amino acid sequences of known MCPs identified the protein encoded by pMCP9 as porcine MPC-2. The 3' untranslated region of pMCP9 was completed by 3' RACE. Northern analysis using RNA from porcine luteal cells and probes specific for porcine MCP-1 and MCP-2 revealed that porcine luteal cells express both MCPs. According to Southern analysis MCP-2, like MCP-1, is specified by a single copy gene.

Wempe et al. (1) demonstrated that monocyte chemoattractant protein-1 (MCP-1) is expressed in the male accessory gland of the bull , whereas MCP-2 and MCP-3 are not. Since expression of all MCPs in bovine PMNLs after stimulation occurs simultaneously, a special control of MCP gene expression in the bovine accessory sex tissue must operate. Characterization of the genes for bovine MCP-1 (3) and MCP-2 (2) and analysis of 5'upstream regulatory sequences will perhaps allow us to understand the differential gene expression of MCP-1 and MCP-2 in bovine seminal vesicle tissue. It was recently reported that porcine luteal cells also express MCP-1 (4), and the question was raised whether porcine luteal cells behave in a similar or different manner to bovine seminal vesicle tissue with respect to MCP expression. In this contribution we show by PCR, cDNA cloning and Northern blot analysis that the luteal cells of porcine corpus luteum (CL) as well as MCP-1 also express MCP-2.

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Material and Methods

Preparation of cDNA from porcine CL followed the protocol described by Wempe et al.(2). A cDNA library generated as reported in λUni-ZapXR (4) was employed. The non-amplified porcine corpus luteum cDNA expression library containing 1.7 $x10^7$ independent clones was subjected to screening by plaque hybridization using a digoxigenin-labeled porcine MCP-1 specific DNA probe as described (4). Nitrocellulose membranes (Sartorius, Göttingen, FRG) were used for plaque hybridization. Hybridization with the digoxigenin-labeled DNA probes was performed according to manufacturer's instructions (Boehringer, Mannheim), followed by washing of filters in 2xSSC containing 0.1% SDS at room temperature and 0.1xSSC containing 0.1% SDS at 68°C. Digoxigenin-positive plaques were visualized by anti-digoxigenin alkaline phosphatase conjugate and the chromogenic substrate BCIP/NBT following a protocol provided by the manufacturer (Boehringer, Mannheim). Positive recombinant pBluescriptSK-plasmids were generated from respective λUni-ZapXR clones by *in vivo* excision. DNA sequences were determined by double strand sequencing using chain termination (5). Internal regions of the insert sequence were obtained employing suitable synthetic primers. Completion of pMCP9, which possessed an incomplete 3'UTR, was achieved by 3'RACE (6). The MCP-2 specific sequence used for 3'RACE was 5'CTGGAGAGCTACACAAG3'. Northern as well as Southern analysis employing GeneScreen membrane (NEN) followed published procedures (7,8,9). Hybridization of DNA blots was performed at 70°C following the protocol of Church and Gilbert (10). Washing conditions were as follows: 2 x SSC and 0.05 % SDS at room temperature for 2 x 10 min each, 2 x SSC and 0.05 % SDS for 2 x 30 min at 70°C and 0.1 x SSC at room temperature for 1 min. DNA probes were [^{32}P]-labeled by random priming to a specific activity of $1x10^9$ cpm/μg employing a commercial kit (Amersham). Northern blots were analyzed by means of a phosphorimager (Molecular Dynamics, model SF). DNA and protein sequence analysis as well as sequence comparisons were performed using the computer program of the University of Wisconsin genetics computer group (11).

Results

Screening of the porcine luteal cell cDNA library with a porcine MCP-1 specific probe furnished three strongly hybridizing λUni-ZapXR clones λMCP5, λMCP7 and λMCP10 as well as the weakly reactive clones λMCP2 and λMCP9. Recombinant pBluescriptSK-derivatives were generated from all λUni-ZapXR clones by *in vivo* excision. Sequencing identified the clones pMCP5, pMCP7 and pMCP10 as being specific for porcine MCP-1 (4). The clones pMCP2 and pMCP9 contained cDNA inserts with DNA sequences differing from those of porcine MCP-1. Clone pMCP9, which contained the longest cDNA insert of 570 bp, was sequenced. pMCP9 possessed an open reading frame (ORF) extending from nt 59 to nt 358. The ATG codon at position 59 was part of a typical consensus sequence for translational start sites (12,13,14). The ORF encoded a protein of 99 amino acid residues which by comparison with the amino acid sequence of bovine MCP-2 yielded 82% sequence identity. Based upon this result we assume that pMCP9 encoded the amino acid sequence for porcine MCP-2.

According to Northern analysis and sequencing, the pMCP9 cDNA sequence possessed an incomplete 3'UTR, which could be completed by 3'RACE (6). The completed cDNA sequence (Fig. 1) was 833 bp long and had 475bp of 3' untranslated region (3'UTR), containing the consensus polyadenylation signal AATAAA, quite similar to the bovine MCP-2 cDNA (2). Starting with nt 567, the 3'UTR possessed an oligo A tract, the length of which

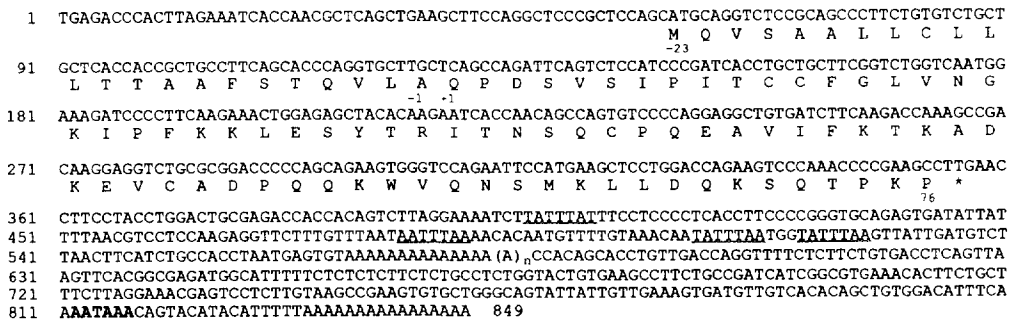

Fig. 1. DNA sequence and derived amino acid sequence of porcine MCP-2. The DNA sequence of pMCP9 comprised nt 1 - 570. The polyadenylation motif is in bold print. The sequence motifs indicative of short-lived mRNA species are underlined. The A residues which could not be precisely defined are indicated in parenthesis. The numbers varied from 1 to 5.

could not be precisely defined in a number of cloned PCR products obtained in 3'RACE experiments. The number of A residues determined varied from 16 - 20.

The 3'-UTR contains four times the motif A/TATTTAA/T, which is responsible for the rapid turnover of an mRNA. The presence of this signal sequence indicates a short half life of an mRNA species (15,16).

Northern analysis was carried out with total RNA from porcine luteal cells, total RNA from porcine CL as well as poly(A+)RNA from porcine CL, employing radioactively labeled probes specific for porcine MCP-1 and MCP-2, respectively. The Northern blot (Fig. 2) clearly indicated that porcine luteal cells express MCP-1 as well as MCP-2 genes. The MCP-2 mRNA was approximately 150bp larger than that of MCP-1. The apparent ratio of mRNAs MCP-1/MCP-2 was determined to be 3 using a phosphorimager.

Southern analysis employing a radioactively labeled pMCP9 probe and porcine genomic DNA restricted with either *Kpn*I, *Bam*HI, *Hind*III or *Pst*I furnished single hybridizing fragments. Only DNA restricted by *Eco*RI yielded two fragments, which can be explained by an *Eco*RI site at nt 312 of the porcine MCP-2 mRNA (Fig. 3). These results indicate that in the porcine species MCP-2 is specified by a single copy gene.

Comparison of amino acid sequences for known MCP-1 and MCP-2 from different mammalian species revealed a high degree of identity for the first 99 residues of human, bovine, porcine and sheep; identity between human and porcine MCP-1 species was highest with 79.8% (Fig. 4). Identification of the family to which the new porcine MCP sequence belongs can be most accurately achieved by comparison of newly detected porcine sequence to the already known bovine and porcine MCP sequences. There are four sequence motifs specific for either MCP-1 or MCP-2 which allow a clear identification of the new sequence as porcine MCP-2 (Fig. 4).

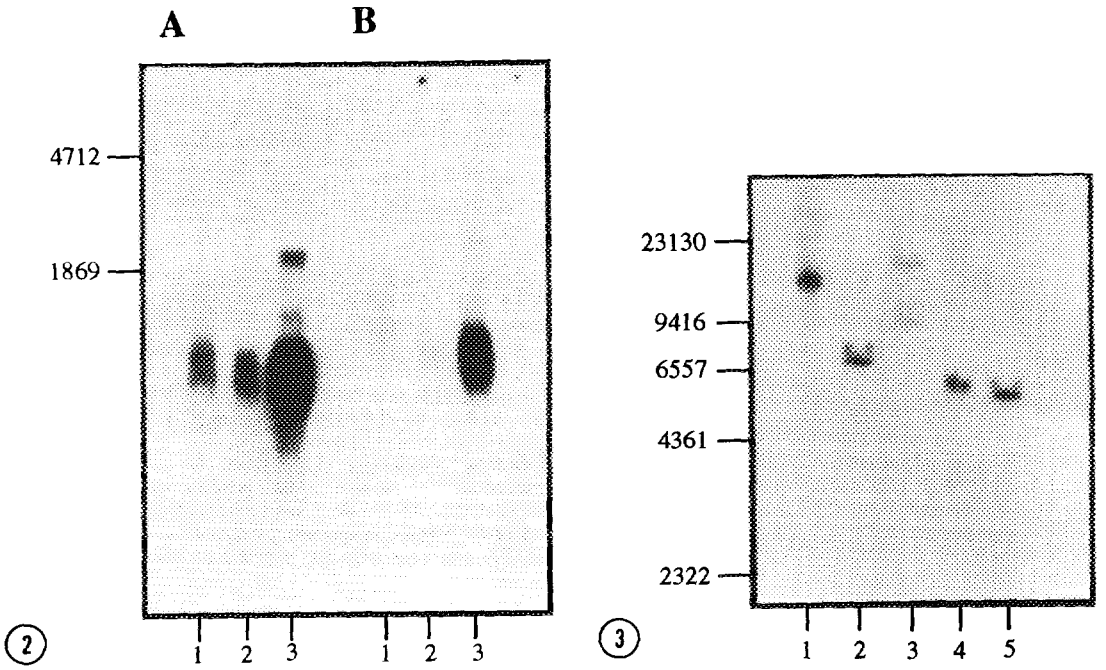

Fig. 2. Gene expression of MCP-1 and MCP-2 in porcine luteal cells. Northern analysis was carried out with total RNA from porcine luteal cells (20 µg; lane 1), porcine CL total RNA (20µg; lane 2) and poly (A+)RNA from porcine CL (1µg; lane 3). A) Hybridization with MCP1-specific cDNA probe. B) Hybridization with MCP-2 specific cDNA probe. The numbers at the ordinate represent marker size in bp.

Fig. 3. Southern blot analysis of the gene for MCP-2. Porcine genomic DNA digested with *Kpn*I (Lane 1), *Bam*HI (lane 2), *Eco*RI (lane 3), *Hind*III (lane 4), and *Pst*I (lane 5). The cDNA insert of pMCP9 was subjected to multiprime labeling and employed for hybridization under stringent conditions. The numbers at the ordinate represent marker size in bp.

Discussion

It has been shown by cDNA cloning as well as Northern analysis that porcine luteal cells express MCP-1 (4) and MCP-2. If RNA from bovine peripheral mononuclear leukocytes (PMNLs) is analyzed by Northern blotting using MCP-specific probes, MCP mRNAs cannot be detected because gene expression of MCP genes in PMNLs requires stimulation (1). Porcine luteal cells are apparently stimulated such that gene expression of the MCP genes occurs. It remains to be shown what kind of stimulation luteal cells are exposed to.

The well established chemotactic potential of MCP-1 and MCP-2 will contribute to the infiltration of monocytes as well as macrophages into the CL and the latter cells very likely participate in luteolysis (20). Further investigations should address the question whether gene expression of MCP genes is a general property of luteal cells in the CL of different mammalian species .

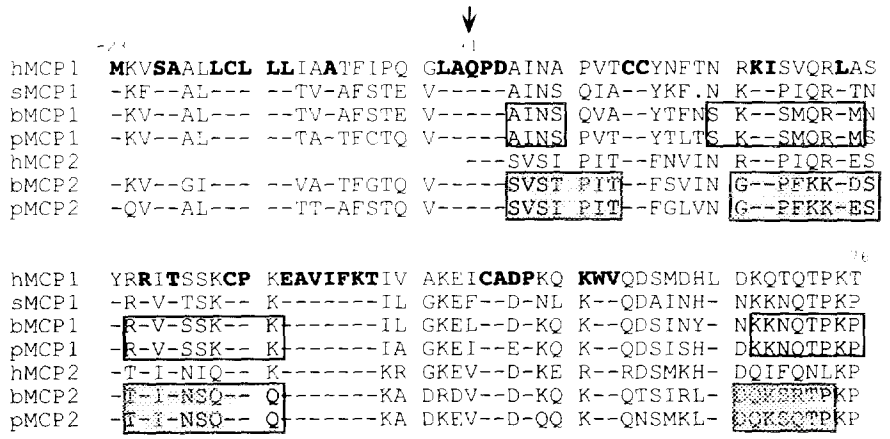

Fig. 4. Comparison of amino acid sequences of human (16), bovine (1), porcine (4), sheep (17) MCP-1 as well as the amino acid sequences of human (18), bovine (2) and porcine MCP-2. The arrow indicates the start of the mature MCP proteins. Amino acid residues identical to the human MCP-1 sequence are represented by dashes. The corresponding residues in the hMCP1 sequence are in bold print. Sequence motifs characteristic for porcine or bovine MCP-1 (no shading) and MCP-2 (shading) are boxed.

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References

1. Wempe, F., Henschen, A. and Scheit, K.H. (1991) DNA Cell Biol. **10**, 671-679.
2. Wempe, F., Hanes, J. and Scheit, K.H. (1994) DNA Cell Biol. **13**, 1-8.
3. Wempe, F., Kleine Kuhlmann, J. and Scheit, K.H. (1994) Biophys. Biochem. Res. Commun. **202**, 1272-1279.
4. Hosang, K., Knoke, I., Klaudiny, J., Wempe, F., Wuttke, W. and Scheit, K.H. (1994) Biochem. Biophys. Res. Commun. **199**, 962-968.
5. Sanger, F., Nicklein, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463-5467.
6. Frohman, M.A., Dush, M.K., and Matin, G.R. (1988) Proc. Natl. Acad. Sci. USA **85**, 8998-9002.
7. Khandijan, E.W. (1986). Mol. Biol. Rep. **11**, 107-115.
8. Southern, E. (1975) J.Mol.Biol. **98**, 503-517.
9. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning (2nd Ed. Cold Spring Harbor Laboratory), Cold Spring Harbor, N.Y.
10. Church, G.M. and Gilbert, W. (1984). Proc. Natl. Acad. Sci. USA **81**, 1991-1995.
11. Devereux, J., Haeberli, P. and Smithies, O. (1984). Nucleic Acids Res. **12**, 387-395.
12. Kozak, M. (1984) Nucleic Acids Res. **12**, 857-872.
13. Kozak, M. (1987) J. Mol. Biol. **196**, 947-950.

14. Lützke, H.A., Chow, H.C., Mickel, F.S., Moss, K.A., Kern, H.F. and Scheele, G.A. (1987) EMBO J. **6**, 43-48.
15. Shawn, G. and Kamen, R. (1986) Cell **46**, 659-667.
16. Caput, D., Beutler, B., Hartog, K., Brown-Shimer, S. and Cerami, A. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1670-1674.
17. Yoshimura, T.N., Yuhki, S.K., Moore, S.K., Appella, E., Lerman, I.M. and Leonard, E.J. (1989) FEBS Lett. **244**, 487-493.
18. Yoshimura, T. (1993) J. Immunol. **11**, 5025-5032.
19. van Damme, J., Proost, P., Lenaerts, J.-P. and Opdenakker, G. (1992) J. Exp. Med. **176**, 59-65.
20. Behrman, H.R., Aten, R.F. and Pepperell, J.R. (1991) in Ovarian Endocrinology (S.G.Hillier, ed.), p190-p225, Blackwell Science Publications, Oxford, UK.