Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 156-162

ISOLATION OF THREE NOVEL POU-DOMAIN CONTAINING cDNA CLONES FROM LACTATING MOUSE MAMMARY GLAND+

Birgit Jehn[1], Gustavo Chicaiza[1], Finian Martin[2] and Rolf Jaggi[1]*

[1]Laboratory for Clinical and Experimental Research, University of Bern, Tiefenaustrasse 120, CH-3004 Bern, Switzerland

[2]Department of Pharmacology, University College Dublin, Dublin 4, Ireland

Received February 17, 1994

Summary: Using a PCR-based cloning strategy to identify putative members of the POU-family of transcription factors from genomic mouse DNA or from cDNA derived from lactating or involuting mouse mammary gland 56 POU-domain containing DNA fragments were isolated. Within these 56 clones three cDNA clones seem to be novel putative members of this transcription factor family, referred to as *mPit-1R*, *mBrn-3R* and *MM-POU-III-A*. Expression pattern studies were performed using a reverse transcriptase-mediated PCR approach. For all three different clones distinct developmental and tissue specific transcript levels were obtained, suggesting a tissue specific function of these newly isolated putative members of the POU-family of transcription factors.

The development of multicellular organisms requires a coordinated and sequential activation and regulation of gene expression which is mediated through the activity of specific transcriptional regulator proteins. Recently a new family of transcription factors could be characterized: the homeobox-related POU-domain containing proteins (1-6). Members of the POU-family of transcription factors are involved in development and differentiation. These proteins are characterized by an evolutionary highly conserved 75-82 amino acid POU-specific domain and a 60 amino acid POU-homeodomain, separated by a variable linker region (7). The POU-specific domain consists of two distinct regions, referred to as the POU_S-A region and the POU_S-B region. The POU-homeodomain comprises three separate helices which form a characteristic helix-turn-helix motif, homologous to products of *Drosophila* homeotic genes or mouse Hox genes (8). Based on primary amino acid-sequence homology studies, a classification of different POU-gene products was established. Five different classes of POU-proteins (POU-I to POU-V) were identified (9, 10).

+The nucleotide sequence data reported in this paper appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers **Z29628** (*mPit-1R*), **Z29629** (*mBrn-3R*) and **Z29638** (*MM-POU-III-A*).

***To whom correspondence should be addressed.** **Fax:** 0041-31-3025933.

0006-291X/94 $5.00

The best characterized member of the POU-I class is ***Pit-1***. *Pit-1* (GHF-1) is a pituitary-specific transcription factor and was shown to be involved in the transcriptional regulation of growth hormone and prolactin genes (1, 11). *Pit-1* mRNA is expressed in the thyrotroph, somatotroph and lactotroph cells of the anterior pituitary gland (1). Recently, the identification of new isoforms of *Pit-1* in somatotroph or thyrotroph-derived cells was reported. *Pit-1a/GHF-2* (13, 14) and *Pit-1T* (15) appear to be splice variants of *Pit-1* gene transcripts, which are expressed in a strictly cell type specific manner. The POU-IV class consists of two characterized members: the mammalian POU-gene *Brn-3* and the *C. elegans* POU-domain containing gene *unc-86*. ***Brn-3*** (*Brain-3*) was originally isolated from rat brain (9) and is highly related with the *C. elegans* gene product *unc-86* (79% amino acid identity). *Brn-3* expression was identified in a variety of different regions of the rat brain and to a lower extent in spleen. Involvement in pattern formation during embryonic development and in general neuronal developmental processes was suggested as possible functional role of *Brn-3* (9). Most of the newly identified members of POU-proteins belong to the POU-III class. The proteins *Brn-1*, *Brn-2* (9), *Brn-4/RSH-2* (16) and *SCIP* (suppressed cAMP-inducible POU; also termed *Oct-6* and *Tst-1*) (9, 17, 18) are the best characterized members of this class of POU proteins. They are all expressed in embryonic and adult brain and in the developing nervous system. These POU-proteins activate gene expression in a tissue-specific manner and they participate in the determination of distinct cell fates.

We have isolated and partially characterized 56 POU-domain containing cDNA clones using a PCR-based cloning approach. Three of them are novel members of this transcription factor family. The three different clones belong to three different classes of POU proteins: ***mPit-1R***, a highly related homologue to *Pit-1*; ***mBrn-3R***, a cDNA clone which shows 81% homology to *mBrn-3* and ***MM-POU-III-A***, which shows a limited homology to all known members of POU proteins belonging to the POU-III class.

Materials and Methods

Animals: Mammary gland tissue from MORO mice was collected from lactating (8 days nourishing) or involuting (5/6 days after removal of the pups from the mother) animals. Tissue samples were immediately polytroned in guanidinium thiocyanate buffer and frozen at -20°C.

Cloning strategy: Total RNA was isolated using guanidinium thiocyanate (19). cDNA was synthesized using 5 µg of poly(A)-selected mRNA from mouse mammary gland, as specified by the manufacturer (cDNA Synthesis System Plus; Amersham). One tenth of the synthesized cDNA or 1 µg of genomic mouse liver DNA were used for amplification with degenerated external primer oligonucleotides (5' GA(G/A)CT(G/T)GAGCA(A/G)TT (T/C)GCC 3' and 5' CTG(G/C)C(G/T)(G/T/C)CGGTT(A/G)CA(A/G)AACCA(A/G/C)AC 3') under the following conditions: 94°C for 1.1 min, 45°C for 1.0 min and 72°C for 3.0 min (30 cycles). Reamplification was performed under the same amplification conditions with internal nested primer oligonucleotides containing two restriction enzyme recognition sites at the 5' end and at the 3' end, respectively (5' GCCGTCTAGATCAGCCA(A/G)A(G/C)(G/C/T)ACCAT(C/T)T 3' and 5' GCCGGGATCCA(T/C)CAC(G/C/T)T(T/C)CTT(T/C)T(T/C)(G/C)A 3'). PCR fragments were subcloned into Bluescript plasmid (Stratagene), electroporated into competent bacterial cells and sequenced on both strands using the dideoxynucleotide chain-termination method (Sequenase Sequencing Kit, Version 2.0, USB).

Analysis of transcript levels by reverse transcriptase-mediated PCR: First strand cDNA was synthesized using 10 μg of total RNA in the presence of gene-specific primer oligonucleotides and Tth-DNA-polymerase (Boehringer Mannheim) according to the instructions of the manufacturer. Second strand cDNA synthesis was followed by DNA amplification by PCR for 30 cycles. 1.0 μl Perfect Match (Stratagene) was added to the amplification mixture. Gene-specific primer oligonucleotides were used to measure expression levels of *mPit-1R*, *mBrn-3R* and *MM-POU-III-A*, respectively. The different primer oligonucleotides had the following sequences: mPit-1R/5' primer: 5' ATGCTTGCAAACTGAAAGCAATT 3'; mPit-R/3' primer: 5' AGAAGGTTTGCTGTGCTCTCT 3'; mBrn-3R/5' primer: 5' GCTGAGAAATCCCACC GCGAG 3'; mBrn-3R/3' primer: 5' GCGATGGCCGCG ATCTTCTC 3'; MM-POU-III-A/5' primer: 5' GCCAGAGTACCATCTGCAGGTC 3'; MM-POU-III-A/3' primer: 5' GACAGCT ACTTCGATAGAAGTCC 3'. Amounts of *mPit-1R*, *mBrn-3R* or *MM-POU-III-A* specific DNA was determined by Southern blot analysis using specific random prime labelled probes.

Results and Discussion

Identification of novel POU-domain containing cDNA clones: Independent rounds of PCR-based cloning experiments were performed to obtain different POU-domain containing cDNA clones from mouse mammary gland or from genomic mouse liver DNA. A total of 882 bacterial clones was analyzed for the presence of inserts. 211 positive cDNA clones were subjected to sequence analysis and a total number of 56 POU-domain containing DNA clones was identified. POU-sequence motifs were identified by comparing the obtained DNA sequences against the complete GenBank and EMBL databases, using the FASTA program (Genetics Computer Group). All five different POU-classes (classes I to V) (9, 10) were represented among the 56 isolated POU-domain containing clones. The distribution of the various POU-domain containing DNA clones among the five groups is summarized in Table 1. POU-classes II, III and IV were predominantly represented. 31 *Oct-1* specific DNA clones were isolated, 10 clones from genomic DNA and 21 clones from cDNA, pointing to a certain preference of the primer oligonucleotides

Table 1. Within the 56 isolated POU-domain containing cDNA clones all five different POU-classes (classes I to V) (9, 10) were represented. Using genomic mouse liver DNA or cDNA derived from lactating mouse mammary gland different POU-domain containing clones were isolated which show a different distribution of POU-classes. Three putative novel members of the POU-family of transcription factors were identified in the cDNA-based PCR cloning approach.

Template DNA	Number of clones isolated		
	Genomic DNA	cDNA	
	Total	Total	New
POU-I class	0	1	1
POU-II class	10	22	0
POU-III class	8	1	1
POU-IV class	8	4	1
POU-V class	2	0	0

for the POU-II class and to higher levels of *Oct-1* transcripts in the synthesized cDNA as compared to the transcript levels of other POU-genes. Among the POU-III class *Brn-4* and *Brn-4* homologous sequences were isolated with highest frequency. The POU-IV class was mainly represented by *Brn-3* specific and *Brn-3* related sequence motifs. Only three members of the POU classes I and V were identified; *Pit-1R* as representative of the POU-I class and *Oct-3* as representative of the POU-V class (Table 1). These results show that with this method of using degenerated primer oligonucleotides members of all five classes of POU-genes could be isolated. Among the 56 isolated clones nine different POU-domain containing DNA fragments were represented, six of them being identical to previously characterized POU-genes and three representing novel members of this gene family.

Three novel POU-family members were identified from cDNA. They belong to three different POU-classes. *mPit-1R* (= *Pit-1 related*) is highly homologous to POU-I class member *Pit-1*. It shows a DNA sequence homology of 88 % to *mPit-1* (Fig. 1A) and 98 % homology at the amino

A

mPIT-1R

```
  1  AGCCAGACGA CCATCTGCCG ATTTGAAAAC TTGCAGCTCA GTTTCAAAAA
 51  TGCTTGCAAA CTGAAAGCAA TTTTATCCAA GTGGCTGGAG GAAGCTGAGC
101  AGGTCGGAGC TTTGTACAAT GAAAAAGTGG GAGCAAACGA AAGGAAGAGG
151  AAACGGAGGA CAACTATCAG TATCGCCGCT AAGGATGCTT TGGAGAGACA
201  CTTTAGAGAG CACAGCAAAC CTTCTTCGCA GGAA
```

B

mBRN-3R

```
  1  TCTAGATCAG CCAAAGTACC ATTTGCAGGT TTGAGTCTCT CACGCTGTCA
 51  CACAACAACA TGATCGCGCT CAAGCCCATC CTGCAGGCGT GGCTGGAGGA
101  AGCTGAGAAA TCCCACCGCG AGAAGCTCAC TAAGCCGGAG CTCTTCAATG
151  GCGCGGAGAA GAAGCGCAAC ACGTCCATCG CGGCGCCGGA GAAGGCCTCT
201  CTGGAAGCCT ACTTCGCCAT CCAGCCAAGG CCCTCCTCGG AGAAGATCGC
251  GGCCATCGCC GAAAAGCTGG ATCC
```

C

MM-POU-III-A

```
  1  TCTAGATCAG CCAGAGTACC ATCTGCAGGT CCGAGGCGCT GCAGCTCAGC
 51  TTCAAAAACA TGTGCAAACT CATGCCATTG CTGAACAAGT GGCTTGAGGA
101  AGCGGACTCT ACGTCCGGCA GTCCCACCAG CTTGGATAAG ATCGCAGCGC
151  AAGGCAGGAG GAGGAAGAAA CGGACTTCTA TCGAAGTAGC TGTCAAATGG
201  T
```

Figure 1. DNA sequences of three novel POU-domain containing DNA clones. Sequences represent parts of the POU-domain, spanning the region from the 5' end of the POU_S-B domain to the 3' end of the POU-homeodomain. **A.** DNA sequence of *mPit-1R*, which shows 88 % to *mPit-1*. **B.** DNA sequence of *mBrn-3R*, which shows 81 % DNA sequence homology to *mBrn-3*. **C.** DNA sequence of *MM-POU-III-A*, which shows a limited sequence homology of 78 % to other POU-III class members.

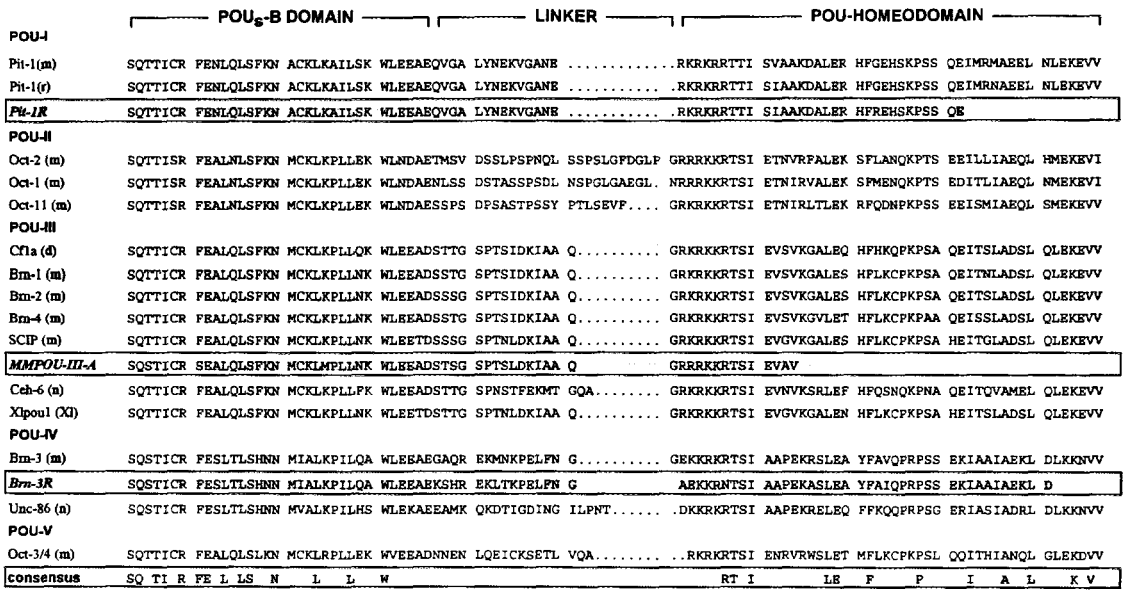

Figure 2. Amino acid sequence alignment of POU-domain containing proteins. Previously characterized and three novel POU-domain sequences were grouped into five different classes, POU-I to POU-V, respectively. m indicates mouse; r, rat; n, *C. elegans* and d, Drosophila. On top, specific regions of the POU-domain are indicated: POU_S-B, the B-subdomain of the POU-specific domain; Linker, a poorly conserved spacer region and POU-homeodomain, containing three distinct helices. At the bottom, the overall conserved consensus sequence is depicted.

acid level, as shown in the amino acid alignments in Figure 2, where the amino acid sequences of several POU-domain containing proteins are shown schematically. *mBrn-3R* is related to *mBrn-3*. It has a DNA sequence homology of 81 % (Fig. 1B) and a amino acid homology of 86 % to *mBrn-3* (Fig. 2). Amino acid exchanges, as compared to the *mBrn-3* sequence, occurred mainly in the evolutionary unconserved linker region, which separates the POU-specific domain and the POU-homeodomain. The third novel family member *MM-POU-III-A* shows a limited DNA sequence homology of 78 % to previously characterized POU-III class members (Fig. 1C). The homology at the amino acid sequence level to POU-III class members varies from 89 % to 95 %, as shown in Figure 2, suggesting that *MM-POU-III-A* is a member of this class of POU-proteins. Again, most amino acid exchanges occurred in the non-conserved linker region of the POU-domain.

Expression levels of *mPit-1R*, *mBrn-3* and *MM-POU-III-A* in different developmental stages of mouse mammary gland and in different mouse organs: To determine the expression levels of the three novel identified POU-family members a reverse transcriptase-mediated PCR approach was used. The distinct expression patterns obtained in different stages of mouse mammary gland development and in a variety of mouse organs are shown in Figure 3. *mPit-1R* expression was detected in all investigated stages of mouse mammary gland development, with highest levels in virgin mammary gland. Additionally, it is strongly expressed in cardiac and skeletal muscle and to a lower extent in brain and liver (Figure 3). *mBrn-3R* expression was

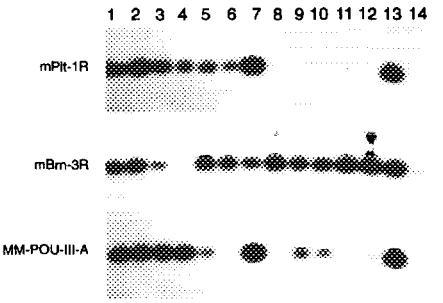

Figure 3. Expression patterns of the three novel POU genes in different mouse organs. Reverse transcriptase-mediated PCR approach: Single stranded cDNA was synthesized using 10 µg of total RNA derived from pubertal (lane 1), virgin (lane 2), pregnant (lane 3), lactating (lane 4) or involuting mouse mammary gland (lane 5) and from brain (lane 6), heart (lane 7), lung (lane 8), kidney (lane 9), spleen (lane 10), liver (lane 11), intestine (lane 12), skeletal muscle (lane 13). A negative control is shown in lane 14. After 30 cycles of amplification a 10 µl aliquot was removed from the reaction and subjected to agagrose gel electrophoresis. The different expression levels were visualized by Southern blot analysis using random prime labelled gene-specific fragments as hybridization probes.

clearly detectable in virgin mice, during puberty and in early involution. Low expression was observed during pregnancy and in lactating mouse mammary glands. In addition, a variable amount of *mBrn-3R* expression was determined in all organs analyzed (Figure 3). Measurement of *MM-POU-III-A* expression revealed relatively high levels in virgin mice, during puberty and pregnancy and in lactation, whereas only a low level was observed during early involution. Highest expression was observed in cardiac and skeletal muscle and lower levels in kidney and spleen (Fig. 3). The distinct expression patterns may suggest a general involvement of these transcription factors in regulatory or differentiation processes and in the determination of specific cell fates. The three cDNA clones described here were isolated from cDNA derived from lactating mouse mammary gland and their distinct transcriptional activity at different stages of mouse mammary gland development point to a possible involvement in regulatory or developmental processes in this gland. However, due to the expression in other organs one would assume a more general role of these transcription factors during development.

Based on further sequence informations it will be possible to investigate the function of these genes in more detail. Such studies would include the characterization of putative DNA-recognition elements of *mPit-1R*, *mBrn-3R* and *MM-POU-III-A* and to identify their localization in promoter regions of specific genes. These studies might elucidate the role and involvement in development and differentiation of the three novel identified POU-genes.

References

1. Ingraham, H. A., Chen, R., Mangalam, H. J., Elsholtz, H. P., Flynn, S. E., Lin, C. R., Simmons, M., Swanson, L., and Rosenfeld, M. G. (1988) Cell 55, 519-529.
2. Fletcher, C., Heintz, N., and Roeder, R. G. (1987) Cell 51, 773-781.

3. Sturm, R. A., Das, G., and Herr, W. (1988) Genes & Dev. 2, 1582-1599.

4. Müller, M., Ruppert, S., Schaffner, W., and Matthias, P. (1988) Nature 336, 544-551.

5. Scheidereit, C., Cromlish, J. A., Gerster, T., Kawakami, K., Balmaceda, C.-G., Currie, R. A., and Roeder, R. G. (1988) Nature 336, 552-557.

6. Finney, M., Ruvkun, G., and Horvitz, H. R. (1988) Cell 55, 757-769.

7. Herr, W., Sturm, R. A., Clerc, R. G., Corcoran, L. M., Baltimore, D., Sharp, P. A., Ingraham, H. A., Rosenfeld, M. G., Finney, M., Ruvkun, G., and Horvitz, H. R. (1988) Genes & Dev. 2, 1513-1516.

8. McGinnis, W., and Krumlauf, R. (1992) Cell 68, 283-302.

9. He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L., and Rosenfeld, M. G. (1989) Nature 340, 35-42.

10. Rosenfeld, M. G. (1991) Genes & Dev. 5, 897-907.

11. Bodner, M., Castrillo, J.-L., Theill, L. E., Deerinck, T., Ellisman, M., and Karin, M. (1988) Cell 50, 267-275.

12. Morris, A. E., Kloss, P. A., McChesney, R. E., Bancroft, C., and Chasin, L. A. (1992) Nucleic Acids Res. 20, 1355-1361.

13. Theill, L. E., Hattori, K., Lazarro, D., Castrillo, J.-L., and Karin, M. (1992) EMBO J. 11, 2261-2269.

14. Haugen, B. R., Wood, W. M., Gordon, D. F., and Rigway, E. C. (1993) J. Biol. Chem. 268, 20818-20824.

15. Le Moine, C., and Young III, W. S. (1992) Proc. Natl. Acad. Sci. USA 89, 3285-3289.

16. Suzuki, N., Rohdewohld, H., Neumann, T., Gruss, P., and Schöler, H. R. (1990) EMBO J. 9, 3723-3732.

17. Monuki, E. S., Kuhn, R., Weinmaster, G., Trapp, B. D., and Lemke, G. (1990) Science 249, 1300-1303.

18. Chomczynski, P., and Sacchi, N. (1987) Analytical Biochemistry 162, 156-159.