Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 412-421

PREFERENTIAL EXPRESSION OF OSTEOCALCIN-RELATED PROTEIN mRNA IN GONADAL TISSUES OF MALE MICE

Masahiro Sato* and Norihiro Tada

Laboratory Animal Center, Pharma Research and Development Division, Hoechst Japan, Limited, Kawagoe, Saitama 350-11, Japan

Received September 2, 1995

SUMMARY: It was recently reported that the mouse genome contains an osteocalcin cluster formed by three genes, OG1, OG2 and ORG, in order from the 5' to the 3' end of the cluster. These three genes exhibit close structural homology. OG1 and OG2 are abundant in bone, but ORG is expressed in nonosteoid tissues, especially in kidney and lung. Previous studies using transgenic mice carrying diphtheria toxin A-chain gene linked to the rat osteocalcin (OG2) promoter have revealed that the OG2 promoter is active not only in osteoblasts, but also in testis. In this study, reverse transcription-polymerase chain reaction was used to study the expression of these three genes in mouse tissues (including testis). Here we report for the first time that mouse ORG is extensively transcribed in male gonadal tissues including seminal vesicle, preputial gland, testis, caput epididymis and cauda epididymis. These findings suggest that ORG may play a role in differentiation and/or maintenance of male gonadal tissues.

Osteocalcin (OC) [also called bone Gla protein (BGP)] is a noncollagenous protein of bone, has a molecular weight of 10 kDa, and three glutamic acid residues that are γ-carboxylated by a specific γ-carboxylase whose action is vitamin K-dependent (1,2). Previous studies have suggested that OC is synthesized exclusively by osteoblasts and odontoblasts, the tooth counterparts of osteoblasts (3,4). OC is secreted in the bone

*Address all correspondence to Dr. Masahiro Sato, Laboratory Animal Center, Pharma Research and Development Division, Hoechst Japan, Limited, 1-3-2 Minamidai, Kawagoe, Saitama 350-11, Japan.
Fax: +81/492-43-4486.

0006-291X/95 $12.00

matrix just after the onset of bone mineralization (5). Because of its structural characteristics and pattern of expression, it is assumed that OC plays a role in bone mineralization (6,7), although its exact function *in vivo* remains unknown.

A gene coding for OC has been cloned and characterized in humans, rat and mouse (8-11). Recent transfection and transgenic experiments using the 5' flanking promoter region of the OC gene have revealed that the OC promoter is active not only in osteoblasts (9,10), but also in other tissues such as brain (12) and platelets (13). Furthermore, Fleet and Hock (14) have shown that OC mRNA can be identified in various nonosteoid tissues (including liver, kidney, lung and muscle) using the ultrasensitive technique of reverse transcription-polymerase chain reaction (RT-PCR).

Several groups recently found that the mouse genome contains two pseudogenes for OC (15-17). According to Desbois *et al.* (17), the mouse OC gene is clustered within a 23-kb span of genomic DNA, and the composite osteocalcin gene 1 (OG1), osteocalcin gene 2 (OG2) and osteocalcin-related gene (ORG) are arranged in the same transcriptional orientation. The coding sequence of OG2 was identical to the published sequence of the mouse OC cDNA (8). OG1 has the same exon-intron structure as OG2, and its coding sequence exhibits 98% homology with the coding sequence of OG2. The 5'-untranslated region of OG1 is 93% homologous to the 5'-untranslated region of OG2 over 1 kb, and it is therefore believed that both genes are duplicated. The ORG gene appeared to have the same exon-intron structure as OG1 and OG2, and its coding sequence exhibits 96% homoloy with that of OG2. A major difference between ORG and the two other genes was a 4-kb DNA fragment located upstream from the initiator that exhibited no homology to any sequences in the two other genes. OG1 and OG2 are expressed only in bone, whereas ORG is expressed in kidney, lung, brain and liver, but not in bone.

Previous studies using transgenic mice carrying the OC-DT-A construct (in which the diphtheria toxin A-chain gene was linked to the 1.1-kb rat OG2 promoter) have revealed that the OG2 promoter is active not only in osteoblasts, but also in the testes and megakaryocytes (18). Given these findings, we decided to examine whether normal male gonadal tissues express OG2 or other OC-related genes. In this study, we used RT-PCR to show that gonadal tissues of male mice contain high levels of ORG mRNA.

MATERIALS AND METHODS

Animals: B6C3F1 (a hybrid of C57BL/6N and C3H/HeN) mice (aged 0.5 and 2 months) were purchased from CLEA Japan, Inc. (Tokyo, Japan). From these mice, long bone, bone marrow, calvaria, brain, kidney, intestine, heart, spleen, thymus, testis, seminal vesicle, preputial gland, caput epididymis, cauda epididymis, lung, skeletal muscle, bladder, uterus, ovary, pancreas and liver were obtained and immediately frozen in liquid nitrogen. The seminal vesicles could not be excised from mice aged 0.5 months, due to their very small size. RNA was isolated by the guanidinium-isothiocyanate method (19).

RT-PCR analysis of specific mRNAs in mouse tissues: cDNA was prepared from total RNA (4μg) by a RT reaction with the following conditions: RT buffer (GIBCO BRL, Grand Island, NY) containing 50mM Tris-HCl (pH 8.3), 75mM KCl, 3mM $MgCl_2$ and 20mM dithiothreitol, 10U RNasin (Promega, Madison, WI), 0.5mM each of dATP, dCTP, dGTP, and dTTP, and 200U reverse transcriptase (GIBCO BRL) in a final volume of 20μl, with incubation for 1h at 42°C. Following RT, samples were heated at 65°C for 10min. The cDNA solution (3μl) was mixed with 17μl of PCR buffer [final concentrations in a 20μl reaction: 10mM Tris-HCl (pH8.3), 50mM KCl, 2.5mM $MgCl_2$, 5pmol each of the forward and reverse primers, 1U *Taq* DNA polymerase (Perkin-Elmer Cetus), and 0.2mM each of dATP, dCTP, dGTP, and dTTP], and was then amplified using a Perkin-Elmer Cetus thermocycler (Model 9600; Norwalk, CT). The PCR reaction cycling conditions were denaturation at 94°C for 1min, annealing at 58°C for 1min, and extension at 72°C for 2min. Preliminary studies showed that use of 23 cycles was preferable, since the amplified products were at the linear stage prior to plateau (data not shown). The PCR primer set for detection of mouse OC mRNA was as follows: sense primer mOC-S, 5'-GAC ACC ATG AGG ACC CTC TCT-3', corresponded to the mouse OC cDNA sequence (8) from nucleotides 8 to 27, while the reverse primer mOC-RV, 5'-GGC TCC AAG GTA GCG CCG GAG-3', was complementary to nucleotides 359-378 of the cDNA (8). The PCR primer set for detection of mouse β-actin mRNA was as follows: sense primer mβA-S, 5'-CTA AGG CCA ACC GTG AAA AGA T-3', corresponded to the β-actin cDNA sequence (20) from nucleotides 415 to 436, while the reverse primer mβA-RV, 5'-CTT CTC TTT GAT GTC ACG CAC G-3', was complementary to nucleotides 704 to 725 of the cDNA (20). Each primer set was designed to produce a PCR product that included an intron-exon border, thereby eliminating the possibility that DNA contamination was responsible for the resulting target products present in the tissue cDNAs. The resulting products (10μl) were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide. Some gels were blotted onto a *GeneScreenPlus* (NEN, Boston, MA) nylon filter membrane for Southern blot hybridization analysis, as described previously (21,22). The probe directed to an amplified region of the OC cDNAs for mouse calvaria was used for

detection of mouse OC mRNA. Quantitation of PCR products formed was conducted by Southern blot analysis of amplified PCR products using a [^{32}P]-labeled probe, as described previously (21,22). Quantitation was carried out by scanning densitometry using a Shimazu dual-wavelength flying-spot scanner (CS-9000).

Sequencing of the PCR-amplified products: PCR products resulting from the amplification of OC mRNAs in cDNAs prepared from calvaria, testis, preputial gland, seminal vesicle, caput epididymis and cauda epididymis of 2-month-old mice were sequenced to ensure that the PCR products corresponded to OG1, OG2 or ORG. The PCR products were first purified from 2% agarose gels. Purified PCR products were inserted into the pBluescript SK(-) cloning vector (Stratagene, La Jolla, CA). Sequencing of the PCR product insert was carried out on Qiagen-preparations of plasmid DNA using primers corresponding to the T3 and T7 recognition sites of the vector. DNA sequences were determined manually using the Sequenase kit (United States Biochemical Corp., Cleveland, OH).

RESULTS

Expression of ORG in male gonadal tissues

With the use of RT-PCR, we were able to detect a number of messages in the total RNA from femoral bone (bone plus bone marrow), calvaria and male gonadal tissues including testis, seminal vesicle, preputial gland, caput epididymis and cauda epididymis of adult (2-month-old) mice (Fig. 1A, upper column). When Southern blot analysis of the PCR amplified products was carried out, other nonosteoid tissues had smaller amounts of OC mRNA (Fig. 1B). Mouse β-actin mRNA was present in a high degree of abundance in each tissue, showing that the RNAs examined had been processed in an intact form (Fig. 1A, lower column). A quantitative comparison of the relative abundance of the various mRNAs was made by measuring the intensities of amplified bands by densitometric scanning. The levels of calvarial and femoral OC mRNAs were 1.1~1.6-fold higher than those of male gonadal tissues. Among the gonadal tissues, preputial gland, seminal vesicle, caput epididymis and cauda epididymis had larger amounts of OC mRNAs than did testis (data not shown). The OC mRNA levels of the other nonosteoid tissues, such as uterus, were however 55~85-fold lower than those of bone and male gonadal tissues. The finding of ubiquitous expression of OC mRNA in nonosteoid tissues other than male gonadal tissues is in agreement with that of Fleet and Hock (14). To our knowledge, ours is the first report that mouse male gonadal tissues synthesize abundant OC mRNAs.

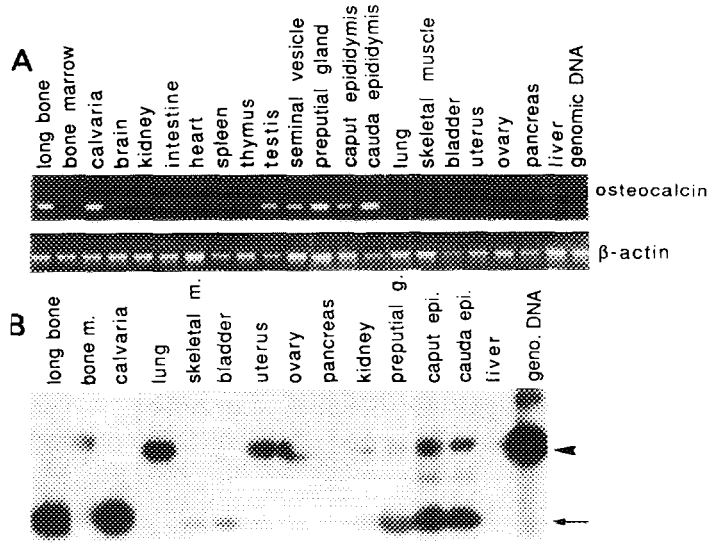

Fig. 1. Identification of OC mRNAs in mouse tissues by RT-PCR. RNAs from various tissues were isolated as described in Materials and Methods. cDNA was prepared and analyzed for OC mRNAs by PCR (23 cycles). PCR products were run on 2% agarose gels containing ethidium bromide (A, upper column). The same amounts of RNA were subjected to cDNA synthesis with the mouse β-actin primer set and processed similar to RT-PCR for OC mRNA (A, lower column). Gels were subsequently analyzed by Southern blot hybridization and radioactive detection using a probe directed to an amplified region of the OC gene (B). The arrow indicates the amplified 165-bp products specific for mouse OC mRNAs. The arrowhead indicates the approximately 660-bp products generated from genomic sequences for OG1, OG2 or ORG. Genomic DNA (1μg) from normal mouse tails was loaded as a negative control.

Sequencing of RT-PCR-amplified fragments of OC mRNAs from male gonadal tissues

To examine which, if any, of the three OC genes (OG1, OG2 and ORG) are expressed in male gonadal tissues, we cloned the RT-PCR products into the pBluescript SK(-) plasmid and performed sequencing. We found some substitution mutations in exons 1 and 2 of these three OC genes (Fig. 2), regions that are amplified by RT-PCR. Typical results are shown in Fig. 3, and the results are summarized in Table 1. cDNAs from calvaria were found to be derived from OG2, whereas those from preputial gland, seminal vesicle, caput epididymis and cauda epididymis were from ORG. In the testis, all the cDNAs examined originated from ORG, although it has been suggested that OG2 is transcribed in mouse testis (18).

```
first exon       *
OG1 GCA GAC ACC ATG AGG ACC ATC TTT CTG CTC ACT 72
OG2 --- --- --- --- --- --- C-- -C- --- --- --- 72
ORG --- --- --- --- --- --- C-- -C- --- --- --- 825

second exon
OG1 AT GCC AAG CCC AGC GGC CCT GAG TCT GAC AAA G 289
OG2 -- --- --- --- --- --- --- --- --- --- --- - 289
ORG -- --- -C- --- -C- --- --- --- --- --- --- - 1042
```

Fig. 2. Nucleotide sequences of the first and second exon regions of OG1, OG2 and ORG, cited from Desbois *et al.* (17). Nucleotides common to the three sequences are indicated by a dash. The asterisk indicates the start codon for translation. Sequence numbers are cited in entirety from Desbois *et al.* (17).

Stage-specific expression of ORG in male gonadal tissues

Total RNAs prepared from gonadal tissues (including testis, seminal vesicle, preputial gland, caput epididymis and cauda epididymis), brain and calvaria of male mice (aged 0.5 and 2 months) were subjected to RT-PCR assay using the mOC-S/mOC-RV primer set. The results are shown in Fig. 4. ORG was extensively transcribed in the early stage of development (0.5 month old) of male gonadal tissues including preputial gland, caput epididymis and cauda epididymis. Age-related increase in the production of OC mRNAs was noted in the testis. Interestingly, the amounts of OC mRNAs expressed in the adult (2-month-old) brain were less than those expressed in the immature (0.5-month-old) brain.

DISCUSSION

In a previous RT-PCR study, ORG was found to be transcribed in kidney and lung in relatively large amounts, in liver and brain in smaller amounts, but not at all in heart, muscle and spleen (17). These findings appear to differ slightly from those of the present study. We detected the presence of cDNAs in all nonosteoid tissues examined, although identification of the resulting cDNAs remains to be performed for some tissues (see Fig. 1). Our findings appear to be consistent with those of Fleet and Hock (14), who detected OC expression in many tissues including liver, kidney, duodenum, lung, brain and muscle, although bone OC mRNA levels were calculated to be 1000-fold higher than duodenal levels. Sequencing of isolated PCR products from rat bone and nonbone (which tissue was not mentioned) cDNAs provided direct evidence that the PCR product obtained was OG2 (14). In a previous study, synthesis of OG2 mRNA was not regulated by 1,25 dihydroxy-vitamin D_3 [1,25 $(OH_2)D_3$] in nonosteoid tissue, although OG2 mRNA in femur increased in response to 1,25 $(OH_2)D_3$ (14). It remains to

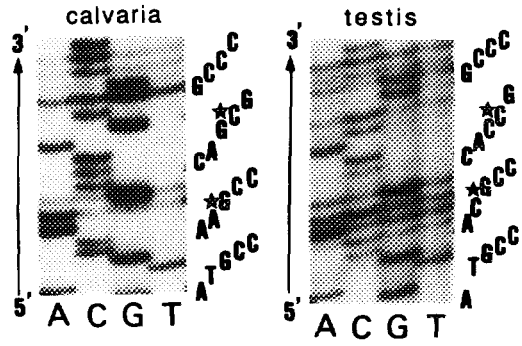

Fig. 3. *Identification of OC mRNAs expressed in mouse tissues by* sequencing of RT-PCR products. The PCR products from testis and calvaria were isolated, subcloned into pBluescript SK(-) and sequenced. Figures show the sequences corresponding to a region of exon 2 of the OC gene family. Asterisks indicate the positions differing between OG2 and ORG (see Fig. 2).

be determined why vitamin D_3-mediated regulation of OG2 expression in nonosteoid tissues differs from that in bone.

Previous studies using transgenic mice carrying the OC-DT-A construct have shown that OG2 promoter is active in testis, since in such mice no mature spermotocytes were observable and Sertoli cells were inactivated or degenerated (18). In the testes studied, however, the interstitial Leydig cells appeared not to have been affected, since they had proliferated (18). OG2 promoter is probably active in either sperm cells or Sertoli cells or both, but not in Leydig cells. In the present study, we failed to detect the presence of OG2 transcripts in

Table 1. Identification of OC genes expressed in male gonadal tissues of 2-month-old mice

Tissue	Type of OC gene[1]
calvaria	OG2 (4/4)[2]
testis	ORG (18/18)
preputial gland	ORG (6/6)
seminal vesicle	ORG (5/5)
caput epididymis	ORG (5/5)
cauda epididymis	ORG (5/5)

[1]OC genes were identified by sequencing the RT-PCR products of the insert in the cloning vector (as shown in Fig. 3).
[2]Parentheses indicate the number of clones identified per number of clones tested.

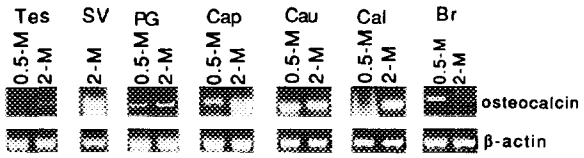

Fig. 4. Developmental regulation of mRNAs from the OC gene family. RNA from testis (Tes), preputial gland (PG), seminal vesicle (SV), caput epididymis (Cap), cauda epididymis (Cau), brain (Br) and calvaria (Cal) of mice [aged 0.5 and 2 months (M)] were isolated as described in Materials and Methods. cDNAs were prepared and analyzed for OC mRNAs by PCR. PCR products were run on 2% agarose gels containing ethidium bromide (upper column). The same amounts of RNA were subjected to RT-PCR for β-actin mRNA (lower column).

normal mouse testis, despite extensive trials (see Table 1), suggesting that expression of OG2 in the testis is much smaller than that of ORG.

In mouse brain, we observed increased expression of cDNAs for OC genes during an immature stage of mouse development (aged 0.5 months), but its expression decreased along aging (see Fig. 4). Kesterson *et al.* (12), using transgenic mice carrying the chloramphenicol acetyltransferase (CAT) gene linked to the human OC promoter, found that the OC promoter is functional in mouse brain, since one of 7 transgenic lines obtained exhibited CAT activity in brain. However, we found no overt abnormalities in brains in a total of 4 OC-DT-A transgenic lines examined (18). This discrepancy could in part be explained by restriction of expression of OG2 to very limited cell populations in mouse brain. It would be interesting to examine what kinds of OC genes are transcribed in mouse brain using the technique we have used here.

At present, the physiological role of ORG in the formation and/or maintenance of mouse male gonadal tissues is unclear. Since ORG has Gla residues like OC, it is conceivable that (i) ORG can bind Ca^{2+} *via* its Gla residues and (ii) male gonadal tissues are rich in vitamin K-dependent γ-carboxylase. It is interesting to note that the presence of Gla-containing proteins has already been reported in human and cock semen (23,24). To our knowledge, no abnormalities in the male gonadal tissues have been reported in vitamin K-deficient animals and in animals treated with warfarin, a vitamin K antagonist (25-27). The production and analysis of ORG-knockout mice would be of great use in assessment of the physiological function of ORG, as previously suggested by Desbois and Karsenty (28).

REFERENCES

1. Poser, J.W., Esch, F.S., Ling, N.C. and Price, P.A. (1980) J. Biol. Chem. 255, 8685-8691.
2. Hauschka, P.V., Lian, J.B., Cole, D.E.C. and Gundberg, C.M. (1989) Physiol. Rev. 69, 990-1047.
3. Price, P.A., Lothringer, J.W., Baukol, S.A. and Reddi, A.H. (1981) J. Biol. Chem. 256, 3781-3784.
4. Lian, J.B., Stein, G.S., Stewart, C., Puchacz, E., Mackowiak, S., Aronow, M., von Deck M. and Shalhoub, V. (1989) Connsect. Tissue Res. 21, 61-69.
5. Liu, F., Malaval, L., Gupta, A.K. and Aubin, J.E. (1994) Dev. Biol. 166, 220-234.
6. Price, P.A., Williamson, M.K., Haba, T., Dell, R.B. and Jee, W.S.S. (1982) Proc. Natl. Acad. Sci. USA 79, 7734-7738.
7. Romberg, R.W., Werness, P.G., Riggs, L.B. and Mann, K.G. (1986) Biochemistry 25, 1176-1180.
8. Celeste, A.J., Rosen, V., Bueker, J.L., Kriz, R., Wang, E.A. and Wozney, J.M. (1986) EMBO J. 5, 1885-1890.
9. Yoon, K., Rutledge, S.J., Buenaga, R.F., Rodan, G.A. (1988) Biochemistry 27, 8581-8526.
10. Lian, J.B., Stewart, C., Puchacz, E., Mackowiak, S., Schalhoub, V., Collart, D., Zambetti, G., Stein, G. (1989) Proc. Natl. Acad. Sci. USA 86, 1143-1147.
11. Theofan, G., Haberstroh, L.M. and Price, P.A. (1989) DNA 8, 213-221.
12. Kesterson, R.A., Stanley, L., DeMayo, F., Finegold, M. and Pike, J.W. (1993) Mol. Endocrinol. 7, 462-467.
13. Thiede, M.A., Smock, S.L., Petersen, D.N., Grasser, W.A., Thompson, D.D. and Nishimoto, S.K. (1994) Endocrinol. 135, 929-937.
14. Fleet, J.C. and Hock, J.M. (1994) J. Bone Miner. Res. 9, 1565-1573.
15. Sztainkrycer, M.D., Dow, D.A., Rowe, D.W. and Pan, L.C. (1993) J. Bone Miner. Res. 8, S121.
16. Rahman, S., Oberdorf, A., Montecino, M., Tanhauser, S.M., Lian, J.B., Stein, G.S. and Stein, J.L. (1993) Endocrinol. 133, 3050-3053.
17. Desbois, C., Hogue, D.A. and Karsenty, G. (1994) J. Biol. Chem. 269, 1183-1190.
18. Tada, N., Ikeda, T., Sato, M., Kitamura, K., Kobayashi, T., Kasai, K. and Hirokawa, K. (1995) Transgenics 1, 649-662.
19. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294-5299.
20. Tokunaga, K., Taniguchi, H., Yoda, K., Shimizu, M. and Sakiyama, S. (1986) Nucleic Acid Res. 14, 2829.
21. Sato, M., Tada, N., Iwase, R. and Amann, E. (1993) Mol. Reprod. Develop. 34, 349-356.
22. Sato, M., Kasai, K. and Tada, N. (1995) Genetic Analysis:Biomol. Engineer. (in press).

23. Gentsch, C. and Martius, C. (1981) Experientia 37, 1261-1263.
24. Soute, B.A.M., Müller-Esterl, W., de Boer-van den Berg M.A.G., Ulrich, M. and Vermeer, C. (1985) FEBS Lett. 190, 137-141.
25. Owen, C.A. and Walter, B.E.J. (1978) Haemostasis 7, 189-202.
26. Price, P.A. and Williamson, M.K. (1981) J. Biol. Chem. 256, 12754-12759.
27. Price, P.A., Williamson, M.K., Haba, T., Dell, R.B. and Jee, W.S.S. (1982) Proc. Natl. Acad. Sci. USA 79, 7734-7738.
28. Desbois, C. and Karsenty, G. (1995) J. Cell. Biochem. 57, 379-383.