Journal of Cellular Biochemistry 54:273–280 (1994)

Isolation of cDNA Clones From an Osteosarcoma-ROS17/2.8 Library by Differential Hybridization

Mary M.Y. Waye and Vincent K.C. Li

Department of Biochemistry, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong (M.M.Y.W.) and Faculty of Medicine, Technical Services Division, University of Toronto, Toronto, Ontario, Canada, M5S 1A8 (V.K.C.L.)

Abstract We have used differential hybridization to isolate and characterize two novel cDNAs expressed in chondrocytes and some osteoblastic cells. A rat osteosarcoma ROS17/2.8 cDNA library was screened and cDNA clones hybridizing strongly to radiolabeled porcine calvaria cDNA but weakly to a control radiolabeled cDNA were isolated. Two clones were obtained—p.6.1 and p.10.15. A radiolabeled probe of p10.15 was shown to hybridize specifically to a 2.3 Kb message RNA from a chondrogenic clonal cell population from rat calvaria–RCJ 3.1C5.18, and the mRNA was downregulated by 1,25 $(OH)_2D_3$, which inhibits chondrogenesis in these cells. The other clone, p6.1, was found to hybridize to a 0.95 Kb message that is expressed in rat liver, kidney, lung, muscle, and brain, but not expressed in spleen and expressed only in low levels in thymus. © 1994 Wiley-Liss, Inc.

Key words: differential hybridization, osteoblastic cDNA, ROS17/2.8, chondrocytes, calvaria

We have chosen a cDNA library [Oldberg et al., 1986] from a well studied osteosarcoma cell line [Noda and Rodan, 1987; Noda et al., 1987] to isolate genes which may be modulated during osteoblastic cell development. Two populations of ^{32}P-labeled cDNA were used to screen replicas of the ROS17/2.8 cDNA library. Pig calvaria cDNA was chosen as a probe for osteoblastic cDNA in a rat library based on the following assumptions: (1) Important osteoblastic genes should be evolutionarily conserved, and therefore pig osteoblastic cDNA should also hybridize to rat osteoblastic cDNA; (2) The lack of rat repetitive sequences [Owens et al., 1985; Shore et al., 1986] in the pig calvaria cDNA should improve the sensitivity of the screening procedure. The replicate filter was hybridized with a negative control using cDNA from a rat calvaria cell line RC 3.2.4.4. This cell line was chosen because it has lost some of the osteoblastic characteristics, for example, collagen type I expression [Aubin et al., 1982; Waye et al., 1989]. Furthermore, the use of a rat cDNA population as a control cDNA should give hybridization signals mostly representative of the rat repetitive DNA. We chose this calvaria cell line instead of a noncalvaria cell line (e.g., a fibroblastic cell line) because we are more interested in the initial events that lead to the formation of bone rather than the final phenotypes that are characteristic of terminally differentiated bone cells. The rat calvaria cell line RC 3.2.4.4 cannot give rise to bone nodules in vitro and has lost the characteristics of early bone stem cells in addition to the loss of collagen type I expression, while the pig calvaria cells contain early stems cells that would give rise to bone cells. Therefore, if we choose a noncalvaria cell line as the negative control, most of the clones isolated by differential hybridization would be characteristic of terminally differentiated bone cells; if we choose a calvaria cell line that had lost the early bone stem cell characteristic, we would increase the ratio of cDNA clones that are important in the early events (that lead to the formation of bone cells) compared to those clones that are characteristic of terminally differentiated bone cells. Thus, the rat calvaria cell line RC 3.2.4.4 was used as the negative control and the pig calvaria cells were used as the positive probe for

Received September 2, 1993; accepted October 21, 1993.

Address reprint requests to Dr. Mary Waye, Department of Biochemistry, Room 302C, Choh-Ming Li Basic Medical Science Building, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong.

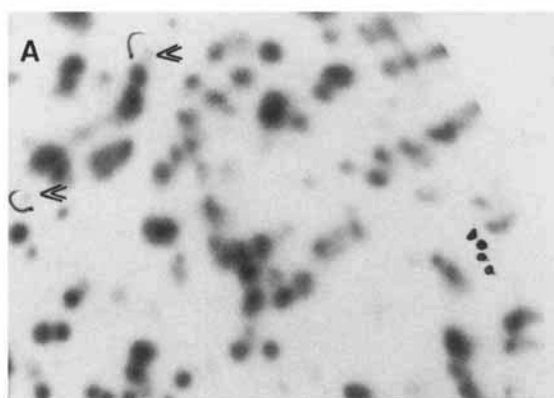
© 1994 Wiley-Liss, Inc.

the generation of ^{32}P-labeled cDNAs. Two clones obtained from such a differential screening experiment were further characterized by DNA sequence and Northern blot analysis.

MATERIALS AND METHODS

Differential Hybridization

The ROS17/2.8 λgt11 cDNA library [Oldberg et al., 1986] was plated at a density of approximately 500 per 13.5 cm^2 plate. A total of 2,500 plaques were screened. Samples of the library were transferred to nitrocellulose (Millipore). Replicas of each filter were then denatured with 0.5 N NaOH, 1 M Tris, pH 7.4, and renatured with 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl. The filters were then dried and baked in a vacuum oven at 80°C for 1 hr. Prehybridization was carried out in 0.5 M $NaPO_4$, pH 7.2, 7% SDS, 1% BSA for 2–3 hr at 67°C and then labeled cDNA probes (10^6 cpm/ml) from pig calvaria or rat osteoblastic cell line 3.2.4.4 were added and hybridized for 24 hr at 58°C. cDNA probes were labeled either by using reverse transcriptase and α-^{32}P dATP [Watson and Jackson, 1985] or by PCR reactions using the forward and reverse primers of the lambda phage [Jansen and Ledley, 1989].



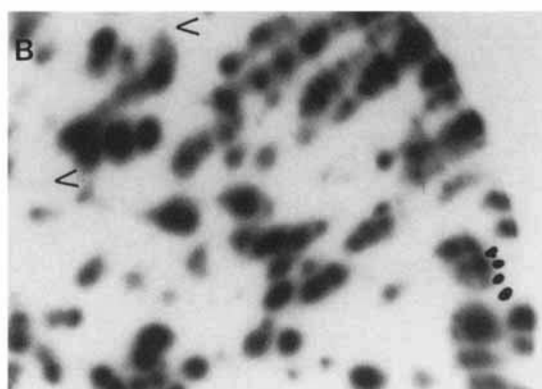


Fig. 1. Replica plaque lifts of lambda gt11 clones from the ROS17/2.8 cDNA library. Arrows indicate clones which hybridized stronger in ^{32}P-labeled fetal porcine calvaria cDNA compared with ^{32}P-labeled rat RC3.2.4.4. cDNA. **A:** The filter was hybridized with ^{32}P-labeled fetal porcine calvaria cDNA. **B:** The filter was hybridized with ^{32}P-labeled rat RC3.2.4.4. cDNA.

Northern Blot Analysis

Total RNA was isolated from either trypsinized or scraped dishes with urea-$LiCl_4$ [Murphy et al., 1986]. For northern blots, 20 μg total RNA was separated by 1% agarose-formaldehyde gel electrophoresis [Fourney et al., 1988]. Random primer-generated probes were labeled with α-^{32}P dCTP to a specific activity of $5 \times 10^8 - 2 \times 10^9$ cpm/mg with a Pharmacia kit, and purified with 5 ml G-50 sephadex columns (Pharmacia). Blots were prehybridized for 2–24 hr, and then hybridized with fresh buffer containing $2–5 \times 10^6$ cpm/ml denatured probes for 16 hr at 42°C. Hybridization buffer consisted of 5× SSC, sodium phosphate 50 mM, pH 6.5, formamide 50%, SDS 0.1%, 5× Denhardt solution, and denatured salmon sperm DNA at 250 μg/ml or tRNA at 100 μg/ml. The filters were washed twice in 2× SSC at room temperature and then twice in 0.1× SSC and 0.1% SDS at 50°C or 65°C.

Southern Blot Analysis

Genomic DNA was isolated from the liver of a rat fasted for 1 d in order to reduce the level of glycogen contamination. Five grams of liver were ground up in a mortar cooled in liquid nitrogen. Ten milligrams of proteinase K in 150 ml of 20% SDS, 0.1 M EDTA, pH 7.4, was added and the mixture was digested at 45°C for 3 hr. The DNA was then phenol extracted for 30 min and ethanol precipitated. High molecular weight DNA was spooled with a 15 ml glass culture tube and the DNA was then rinsed three times in 330 ml of 70% EtOH for 20 min each. The final wash was done with 95% EtOH and the dried DNA was then dissolved overnight at 4°C in 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA.

Twenty micrograms of DNA was separated by 1% agarose-TBE gel electrophoresis and the gel was treated with 0.25 N hydrochloric acid for 8 min, rinsed briefly with water, then soaked in 1 M NaCl/0.5 M NaOH 2 × 15 min with agitation; the gel was neutralized by soaking in 0.5 M

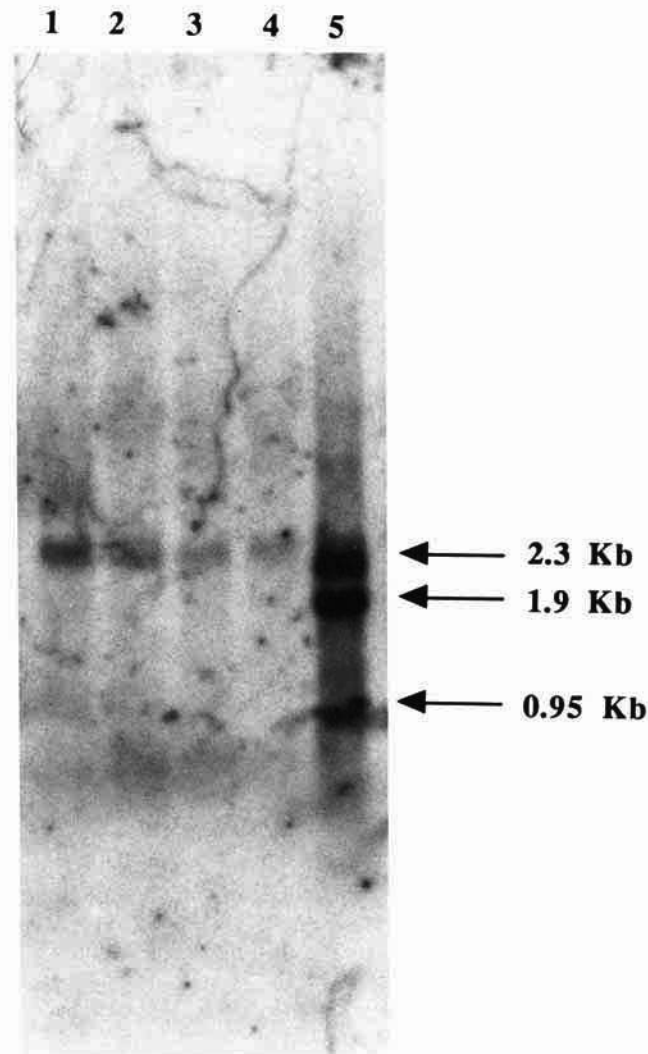


Fig. 2. Northern blot analysis with the λ10.1 probe. Lane 1, 20 μg of total RNA from a chondrogenic clonal cell population from rat calvaria–RCJ3.1C5.18 treated with 24,25 $(OH)_2D_3$; lane 2, 20 μg of total RNA from a chondrogenic clonal cell population from rat calvaria–RCJ3.1C5.18; lane 3, 20 μg of total RNA from RCJ3.1C5.18 treated with 1,25 $(OH)_2D_3$; lane 4, 20 μg of total RNA from a rat osteoblastic cell population; lane 5, 5 μg of poly A^+ RNA from a human osteosarcoma cell line MG 63.

Tris-HCl, pH 7.4/1.5 M NaCl, pH 7.0, 2 × 15 min, and transferred to a nylon membrane (ICN) using standard methods [Maniatis et al., 1982]. The nylon filter was hybridized using conditions similar to those described under Northern blot analysis.

DNA Sequence Analysis

DNA inserts from the lambda gt11 clones were amplified using polymerase chain reaction (with primers having sequences that are the same as the BioLab lambda primers). The amplified DNA was then digested with *Eco*RI and cloned into the phagemid pT7T3 (Pharmacia, Toronto). Single-stranded DNA was prepared and sequenced using the modified T7 DNA polymerase (Pharmacia, Toronto). A nested set of deletional mutants were constructed using the exonuclease III method [Henikoff, 1987]. The DNAstar program was used for aligning the sequences and the FASTA program was used to search for homologous sequences in the GenBank and EMBL data banks [Pearson and Lipman, 1988]. Searches of the Swiss protein bank and the translated version of GenBank were performed with the FASTP program. The BLASTN program [Altschul et al., 1990] was also used to check the NCBI (National Center for Biotechnology Information) nonredundant database.

RESULTS

Differential Hybridization

A rat osteosarcoma ROS17/2.8 cDNA library was screened with labeled cDNA from pig calvaria or RC3.2.4.4 cells. One cDNA clone (λ10.1) showed a high level of radioactivity when hybridized with pig calvaria ^{32}P-labeled cDNA but a lower signal with ^{32}P-labeled rat RC 3.2.4.4. cDNA (Fig. 1). The clone was isolated and then subcloned into a phagemid vector pT7T3 for preparation of DNA probes in Northern analysis and DNA sequencing experiments. Although the *plaques that* were selected do not give a strong signal with the pig calvaria cDNA probe, they were chosen instead of those that give strong signals because plaques that give strong signals probably carry cDNAs that code for abundant structural proteins rather than novel regulatory proteins.

Northern Blot and Southern Blot Analysis

The DNA insert from clone λ10.1 was amplified with primers in the lambda phage arms region in the presence of ^{32}P and used as probes

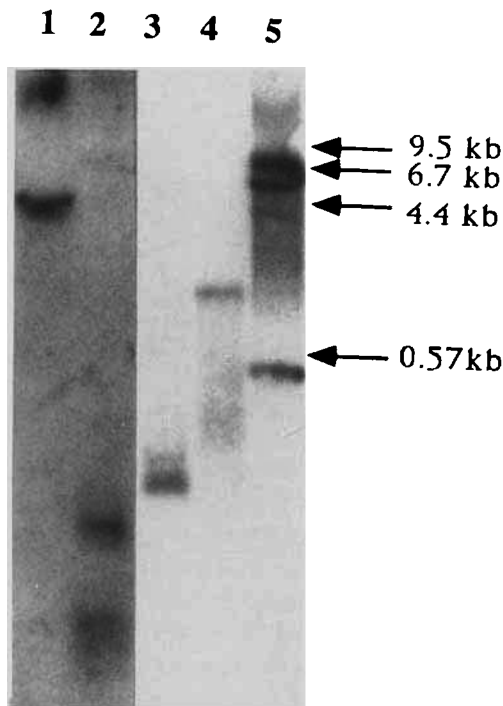


Fig. 3. Southern blot analysis with the p10.15 (lanes 1 and 2) or with the p6.1 probe (lanes 3 and 4). Twenty micrograms of rat genomic DNA was digested with either *Eco*RI (lanes 1 and 3) or with *Bam*HI (lanes 2 and 4) and then probed with the p6.1 probe (lanes 3 and 4); the blot was then boiled in distilled water for 20 min to remove the radioactive p6.1 probe and reprobed with p10.15 (lanes 1 and 2). The marker used was *Hin*dIII-digested lambda DNA (lane 5).

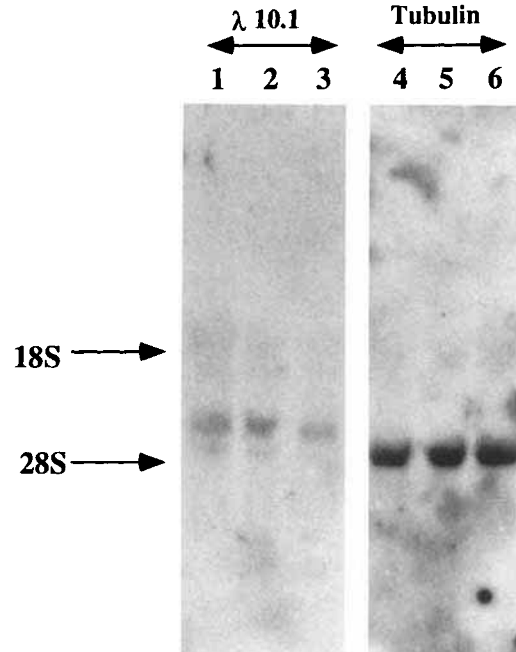


Fig. 4. Northern blot analysis with the λ10.1 probe. Lanes 1 and 6, RNA from a chondrogenic clonal cell population from rat calvaria–RCJ3.1C5.18 treated with 24,25 $(OH)_2D_3$; lanes 2 and 5, control untreated RCJ3.1C5.18; lanes 3 and 4, RCJ3.1C5.18 treated with 1,25 $(OH)_2D_3$. The blot was either probed with the λ10.1 probe (lanes 1 to 3) or a tubulin probe (lanes 4 to 6).

for Northern blot analysis. In a chondrogenic clonal cell population from rat calvaria—RCJ 3.1C5.18 [Grigoriadis et al., 1990] and in a rat osteoblastic population RCIII [Bellows et al., 1986], only one band of molecular weight 2.3 Kb was observed (lanes 2 and lane 4 of Fig. 2, respectively). More than one band was observed in the RNA sample of a human osteosarcoma cell line MG63. They are of molecular weight 2.3 Kb, 1.9 Kb, and 0.95 Kb (lane 5 of Fig. 2). The minor difference in mobility between the 2.3 band in lanes 1–4 compared to that of lane 5 is either due to species difference (lanes 1–4 represent mRNA obtained from rat cells whereas lane 5 represents mRNA obtained from human cells), or it could be due to the fact that different amounts of mRNA were loaded (lanes 1–4 were loaded with 20 μg of total mRNA whereas lane 5 was loaded with 5 μg of poly A$^+$ mRNA). To distinguish whether the multiple bands observed in the RNA sample of the human osteosarcoma cell line MG63 were due to different messages hybridizing the same cDNA or different cDNAs in the lambda clone λ10.1, we subcloned the DNA insert from λ10.1 into phagemid pT7T3 so that the cDNAs can be further characterized. Two types of phagemids (p10.15 and p6.1) were

TABLE I. Regulation of p10.15 by $1,25(OH)_2D_3$*

Cells used	Time	Levels of p10.15	Average	(SD)
RCJ3.1C5.18 (chondrogenic)	48 hr	51%, 54%	52.5%	(1.6%)
ROS17/2.8 (osteoblastic)	48 hr	81%, 72%	76.5%	(6.4%)
ROS17/2.8 (osteoblastic)	6 d	31%, 32%	31.5%	(0.7%)

*Northern blot analysis of mRNA samples from different cells using ^{32}P-labeled p10.15 as the probe. The signals from the autoradiographic film were scanned by a densitometer and then normalized against the signals from the control lanes (untreated cells in which no $1,25(OH)_2D_3$ was added at time zero). In each case, two independent measurements from two different Northern blots were done for each mRNA sample. SD = standard deviation.

obtained from digesting λ10.1 with *Eco*RI and subcloning the fragments into pT7T3. When DNA inserts of phagemid p10.15 and phagemid p6.1 were used to probe rat genomic DNA digested with *Eco*RI or *Bam*HI, different patterns of hybridization was observed. Phagemid p10.15 gave a 6.4 Kb band with *Eco*RI-digested DNA and a 0.1 Kb and 0.01 Kb band with *Bam*HI-digested DNA; whereas a probe made from phagemid p6.1 gave a 1.7 Kb band with *Eco*RI-digested DNA and a 0.14 Kb band with *Bam*HI-digested DNA (lanes 1–4, respectively, of Fig. 3). Our results suggest that p10.15 and p6.1 are two distinct cDNAs that are present in one single lambda recombinant phage. However, there is a small but definite possibility that the two different probes gave a difference in banding pattern of the Southern blot pattern by coincidence. In order to show more definitively that the two *Eco*R1 fragments are from two different cDNAs, Northern blot analysis was performed. Northern blot analysis using p10.15 shows only the 2.3 Kb band (data not shown), while Northern blot analysis using p6.1 shows only the 0.95 Kb mRNA (as described in the last paragraph of the Results section). The reason that the 0.95 Kb band is not detectable in lanes 1–4 in Figure 2 is that the relative amount of 0.95 Kb message is much less than that of the 2.3 Kb message; the 0.95 Kb band is detectable either with poly

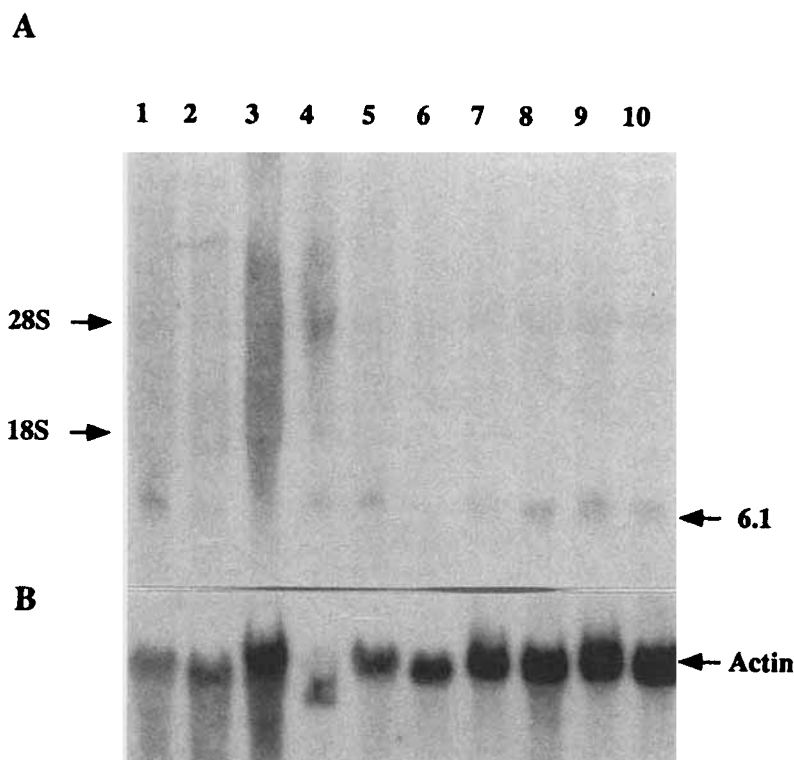


Fig. 5. Northern blot analysis with the p6.1 probe. Lanes 1 and 3, RNA from an osteoblastic cell line ROS17/2.8 treated with $1,25\ (OH)_2D_3$ for 48 hr and 6 d, respectively; lanes 2 and 4, control untreated ROS17/2.8. Lanes 5–9 are RNA samples from thymus, brain, muscle, spleen, and lung, respectively. The blot was either probed with the p6.1 probe (**A**) or an actin probe (**B**).

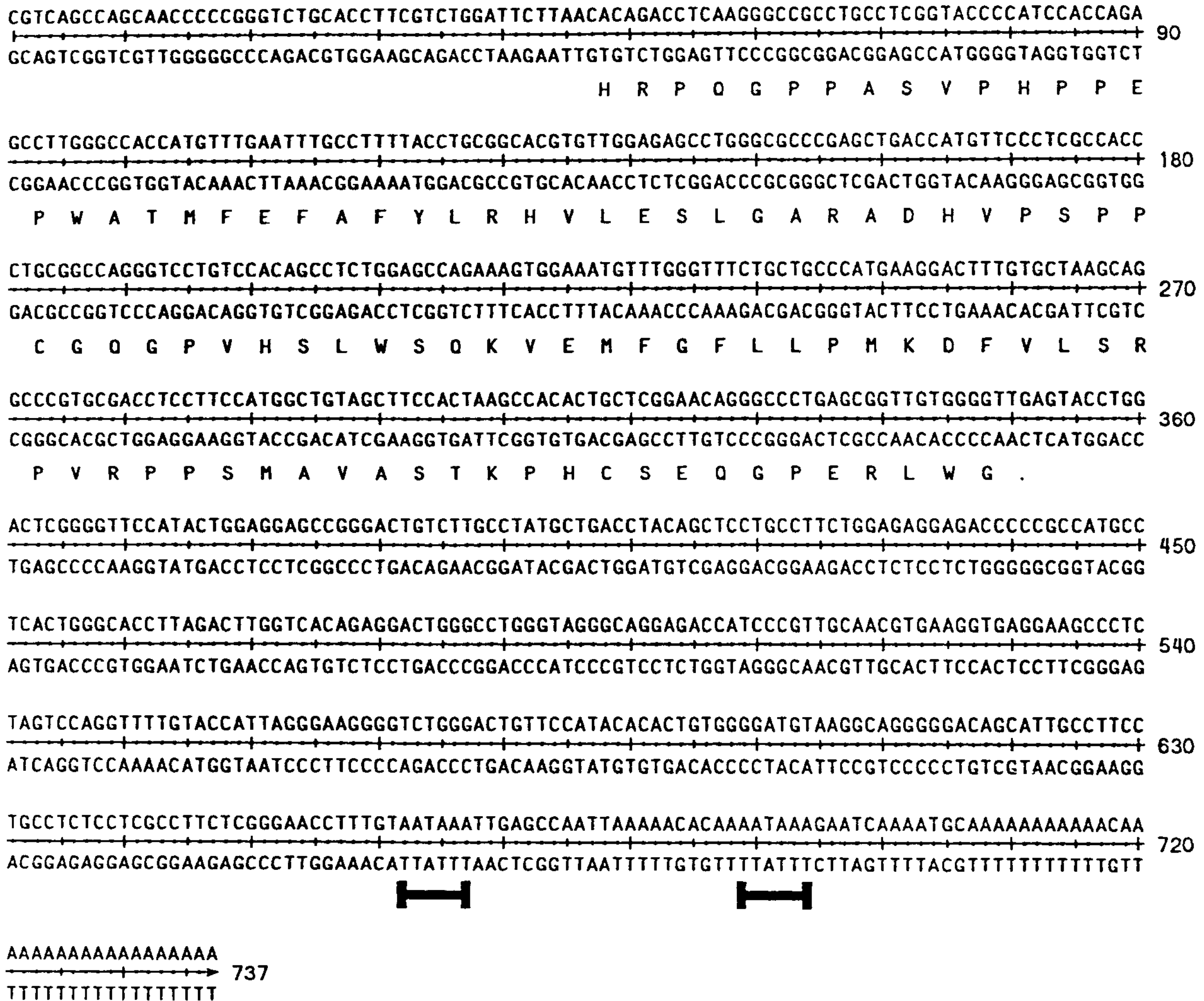


Fig. 6. DNA sequence of p10.15. The bar below the sequence indicates putative polyadenylation signal.

A+ message (lane 5 of Fig. 2) or alternatively, upon prolonged exposure (> 10 d) of the Northern blot (see Fig. 5).

Regulation of the 2.3 Kb Band by 1,25 $(OH)_2D_3$

The 2.3 Kb band can be downregulated by 1,25 $(OH)_2D_3$ (compare lane 3 with the control, lane 2 of Fig. 2); whereas 24,25 $(OH)_2D_3$ has no effect on the regulation of the 2.3 Kb band (compare lane 1 with the control, lane 2 of Fig. 2). To confirm that the difference in the levels of the 2.3 Kb band was not due to differences in loading, we repeated the Northern blot experiment (Fig. 4, left) and reprobed the blot with a tubulin probe (Fig. 4, right). To measure the change in the levels of the 2.3 Kb message with 1,25 $(OH)_2D_3$ treatment, the Northern blot experiments were repeated (lanes 1–3 of Figs. 2 and 4) and the signals were scanned with a densitometer. Table I shows that the change in the levels of the 2.3 Kb message with 1,25 $(OH)_2D_3$ treatment is statistically significant.

Tissue Distribution of the 2.3 Kb and 0.95 Kb mRNAs

p10.1 and p6.1 were used to probe RNA obtained from different tissues. It can be shown that the p10.15 is expressed in all the tissue tested (data not shown) while the 0.95 Kb mRNA detected by p6.1 is expressed in liver, kidney, lung, muscle, and brain, but not expressed in spleen and expressed in low levels in thymus (Fig. 5).

DISCUSSION

The DNA sequences of p10.15 (Fig. 6) and p6.1 (Fig. 7) were obtained by the di-deoxy sequencing method [Sanger et al., 1977]. The longest open-reading-frame of p10.15 was deduced from the nucleotide sequence and was used to

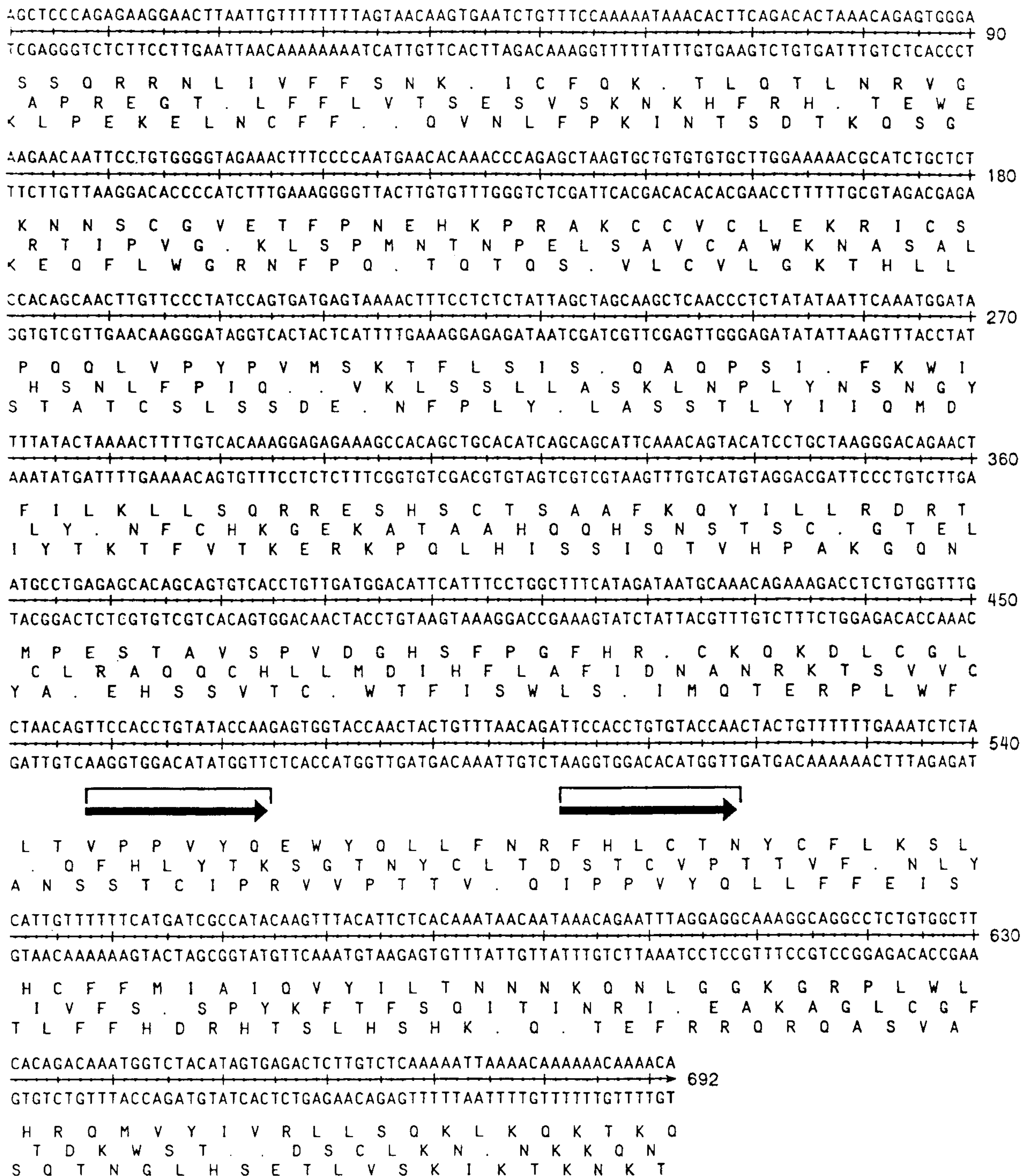


Fig. 7. DNA sequence of p6.1. The bar below the sequence indicates putative polyadenylation signal. The arrows indicate a direct repeat of 17 base pairs.

search the translated sequence of the GenBank. The match with the highest score revealed that there is a 47.6% identity in a 21 amino acid stretch with the rat nuclear receptor coregulator or RXRβ [Yu et al., 1991]. However, this open-reading-frame does not have the zinc-finger domain similar to RXRβ, and thus this marginal degree of homology with RXRβ is probably due to a chance event. In an attempt to find out if this protein has a leucine zipper like that of c-*fos* or c-*jun* [Ransone and Verma, 1990], a helix wheel of the amino acid region which has homology with RXRβ was studied. This region lies in an alpha helix region, as predicted by the

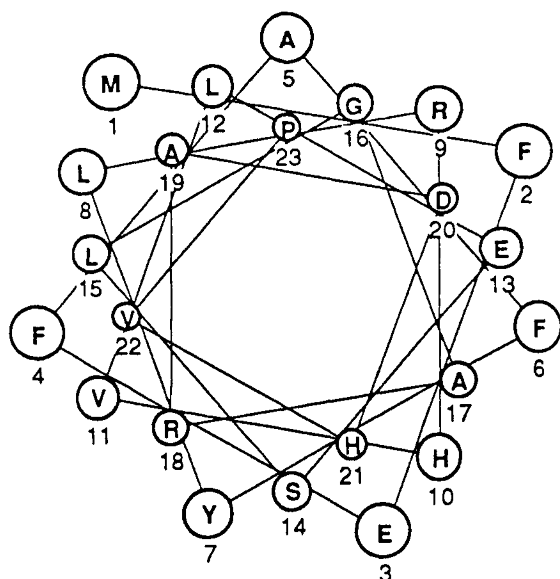


Fig. 8. Helical wheel analysis of the second alpha helix of the putative open-reading-frame of p10.15. The second alpha helix of the putative protein was analyzed using the DNAstar program. The amino acids Phe4, Leu8, Leu12, and Leu15 form a hydrophobic surface.

Chou-Fasman method. The alpha helix shows a hydrophobic surface with three leucine and one phenylalanine (Fig. 8). Although this differs from the leucine zipper of c-*jun* or c-*fos*, this hydrophobic surface might be important for self-dimerization or heteromer formation with other proteins.

In conclusion, we have isolated two novel cDNAs from the rat osteosarcoma cDNA library: p10.15 is expressed in all the tissue tested, whereas p6.1 is expressed in most tissue except spleen and thymus. Northern blot analysis revealed that p10.15 can be downregulated by 1,25 $(OH)_2$ vitamin D_3 in two different cell types: RCJ 3.1C5.18, a clonal rat chondrogenic cell population, and ROS17/2.8, a rat osteoblastic cell line. Both cDNAs were partially sequenced and searches with the GenBanks did not reveal any significant match with published sequences.

ACKNOWLEDGMENTS

We thank Dr. Oldberg for providing the ROS17/2.8 cDNA library, Dr. J.E. Aubin for providing the rat calvaria cells RC 3.2.4.4., and K.L. Lee and Dr. A. Gupta for providing mRNA samples. This study was supported by a group grant from the Medical Research Council of Canada.

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990): Basic local alignment search tool. J Mol Biol 215:403–410.

Aubin JE, Heersche JNM, Merrilees MJ, Sodek J (1982): Isolation of bone cell clones with differences in growth, hormone responses, and extracellular matrix production. J Cell Biol 92:452–461.

Bellows CG, Aubin JE, Heersche JNM, Antosz ME (1986): Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif Tissue Int 38:143–154.

Fourney RM, Miyakoshi J, Day RS, Paterson MC (1988): Northern blotting: Efficient RNA staining and transfer. Focus 10:5–7.

Grigoriadis AE, Heersche JNM, Aubin JE (1990): Continuously growing bipotential and monopotential myogenic, adipogenic, and chondrogenic subclones isolated from the multipotential RCJ 3.1 clonal cell line. Dev Biol 142:313–318.

Henikoff S (1987): "Unidirectional Digestion With Exonuclease III in DNA Sequence Analysis." San Diego: Academic Press, Meth in Enym 155:156–165.

Jansen R, Ledley FD (1989): Production of discrete high specific activity DNA probes using the polymerase chain reaction. Gene Anal Tech 6:79–83.

Maniatis T, Fritsch EF, Sambrook J (1982): "Molecular Cloning. A Laboratory manual." New York: Cold Spring Harbor Laboratory Press.

Murphy WJ, Watkins KP, Agabian N (1986): Identification of a novel Y branch structure as an intermediate in trypanosome mRNA processing: Evidence for *Trans* splicing. Cell 47:517–525.

Noda M, Rodan GA (1987): Type β transforming growth factor (TGFβ) regulation of alkaline phosphatase expression and other phenotype-related mRNAs in osteoblastic rat osteosarcoma cells. J Cell Physiol 133:426–437.

Noda M, Yoon K, Thiede M, Buenaga R, Weiss M, Henthorn P, Harris H, Rodan GA (1987): cDNA cloning of alkaline phosphatase from rat osteosarcoma (ROS 17/2.8) cells. J Bone Miner Res 2:161–164.

Oldberg A, Franzen A, Heinegard D (1986): Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Proc Natl Acad Sci 83:8819–8823.

Owens GP, Chaudhari N, Hahn WE (1985): Brain "identifier sequence" is not restricted to brain: Similar abundance in nuclear RNA of other organs. Science 229:1263–1265.

Pearson WR, Lipman DJ (1988): Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444–2448.

Ransone LJ, Verma IM (1990): "Nuclear Proto-oncogenes *FOS* and *JUN*." Palo Alto, California: Annual Reviews Inc.

Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Shore SK, Bacheler LT, Kimball de Riel J, Barrows LR, Lynch M (1986): Cloning and characterization of a rat-specific repetitive DNA sequence. Gene 45:87–93.

Watson CJ, Jackson JK (1985): "An Alternative Procedure for the Synthesis of Double-Stranded cDNA for Cloning in Phage and Plasmid Vectors." Oxford: IRL Press.

Waye MMY, Robinson R, Orfanides AG, Aubin JE (1989): Loss of type I collagen gene expression in rat clonal bone cell lines is accompanied by DNA methylation. Biochem Biophys Res Commun 162:1446–1452.

Yu VC, Delsert C, Anderson B, Holloway JM, Devary OV, Määr AM, Kim SY, Boutin J-M, Glass CK, Rosenfeld M (1991): RXRβ: A coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell 67:1251–1266.