Characterization of the Rat Osteocalcin Gene: Stimulation of Promoter Activity by 1,25-Dihydroxyvitamin D_3

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ABSTRACT: Osteocalcin is an abundant noncollagenous protein in bone, and its synthesis is stimulated by 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. In this study, the rat osteocalcin gene was isolated, sequenced, and found to be a single-copy gene that is highly conserved between human and rat. Northern blot analysis of RNAs from a number of rat tissues revealed osteocalcin mRNA only in calvariae, consistent with bone-specific expression of osteocalcin. In order to investigate promoter activity and its modulation by 1,25$(OH)_2D_3$, plasmids containing the osteocalcin promoter region linked to the reporter enzyme bacterial chloramphenicol acetyltransferase (CAT) were used to transfect rat osteosarcoma ROS 17/2.8 cells, which express osteocalcin endogenously, and UMR 106 cells, which lack osteocalcin expression. Transfected ROS 17/2.8 cells exhibited a higher basal CAT activity than UMR 106 cells. Moreover, 1,25$(OH)_2D_3$ stimulated the CAT expression 5–10-fold only in ROS 17/2.8 cells and not in UMR 106 cells. By use of unidirectional deletion analysis, a domain strongly responsive to 1,25$(OH)_2D_3$ was identified between bases −1035 and −871 upstream from the site of transcription initiation, while a weakly responsive region was found further downstream.

Osteocalcin [bone γ-carboxyglutamic acid (Gla)[1] containing protein (BGP)], synthesized by osteoblasts, is the most abundant noncollagenous protein in adult bone [for reviews, see Lian and Gundberg (1988) and Hauschka (1986)]. Although the physiological role of osteocalcin is not known, serum levels of osteocalcin correlate well with bone turnover (Price, 1983) and have been used as a clinical indicator of bone metabolism (Lian & Gundberg, 1988). Osteocalcin expression appears to be regulated in a developmental-stage-specific and a cell-specific manner. Osteocalcin is expressed relatively late in bone development as demonstrated by radioimmunoassay (Otawara & Price, 1986) and by mRNA quantitation (Yoon et al., 1987). Of the two well-characterized rat osteosarcoma-derived osteoblastic cell lines, ROS 17/2.8 and UMR 106, osteocalcin expression is detected only in ROS 17/2.8 (Fraser et al., 1988). Otherwise these cell lines exhibit similar osteoblastic phenotypes including synthesis of collagen type I and a high level of alkaline phosphatase activity and parathyroid hormone stimulated adenylate cyclase activity (Majeska et al., 1980; Partridge et al., 1981). Both cell lines contain 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] receptors (Price & Baukol, 1980; Partridge et al., 1980).

1,25$(OH)_2D_3$ stimulates osteocalcin synthesis in osteoblastic cells in culture (Price & Baukol, 1980; Lian et al., 1985; Spiess et al., 1986), and its effect is blocked by inhibitors of RNA polymerase II (Pan & Price, 1984), suggesting that it acts at the level of transcription. Similar to other steroid hormone receptors, the 1,25$(OH)_2D_3$ receptor contains a steroid binding region and a DNA binding domain which is suggested to be involved in transcriptional regulation (Burmester et al., 1988; Baker et al., 1988). 1,25$(OH)_2D_3$ controls the expression of several other genes including collagen type I (Kream et al., 1986), matrix-Gla protein (Fraser et al., 1988), parathyroid hormone (Okazaki et al., 1988), c-myc oncogene (Simpson et al., 1987), and a family of calcium binding proteins termed calbindins (Minghetti et al., 1988; Perret et al., 1988). However, the cis-acting DNA sequences involved in the transcriptional regulation by 1,25$(OH)_2D_3$ have not yet been identified.

This study describes the isolation and characterization of the rat osteocalcin gene and the expression of osteocalcin mRNA in various tissues. Promoter activity and its regulation by 1,25$(OH)_2D_3$ were investigated by transient transfection of ROS 17/2.8 and UMR 106 cells, with plasmids containing the promoter region linked to the bacterial chloramphenicol acetyltransferase (CAT). A domain strongly responsive to 1,25$(OH)_2D_3$ was identified in the 5′-flanking region of the osteocalcin gene by using unidirectional deletion analysis.

Materials and Methods

Isolation and Sequencing of Rat Osteocalcin Genomic DNA. An oligonucleotide (5′-AGAGAGAGGGTCCTC-ATGGTGTCTGCTAGGTCTGCACCGAGTTGC-3′) was synthesized corresponding to the complementary sequences of the bases 1–45 of the rat osteocalcin cDNA (Pan & Price, 1985). The 5′-end-labeled oligonucleotide was used to screen aliquots of a rat genomic library (Clontech, Palo Alto, CA), constructed by insertion of rat genomic DNA partially digested with *Eco*RI into the *Eco*RI sites of Charon 4A. Phage DNA was isolated by immunoabsorption to lamdasorb (Promega Biotec, Madison, WI), digested with *Eco*RI, and subcloned into pBS/M13$^+$ (Bluescript, Stratagene, La Jolla, CA). Restriction fragments of the genomic inserts were subcloned into M13mp18 and M13mp19 and sequenced by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (United States Biochemicals, Cleveland, OH). DNA sequences were analyzed by using the University of Wisconsin computer program. All restriction enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Construction of CAT Plasmids. A 1.2-kb *Eco*RI–*Ava*I fragment that contains the 5′-flanking region of the rat os-

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[1] Abbreviations: bp, base pair; CAT, chloramphenicol acetyltransferase; Cm, chloramphenicol; DEAE, diethylaminoethyl; Gla, γ-carboxyglutamic acid; kb, 1000 bp; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ROS, rat osteosarcoma; SDS, sodium dodecyl sulfate; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate.

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teocalcin gene was made blunt ended by using the Klenow fragment of DNA polymerase I, ligated to *Hin*dIII linkers, and inserted into the *Hin*dIII site of the promoterless plasmid pSVOCAT, which was generously provided by Dr. Bruce Howard (NIH, Bethesda, MD). This plasmid, pOCCAT, contains the CAT gene at the 3′ side of the putative osteocalcin promoter region. To obtain deletions at the 5′ end of the genomic fragment, pOCCAT was digested with *Kpn*I and *Eco*RV, incubated with *Exo*III nuclease for 1, 2, 3, and 4 min at 23 °C, and made blunt ended by mung bean nuclease. The ligated DNAs were used to transform *Escherichia coli* DH5α cells (BRL, Gaithersburg, MD). Deletions were characterized by restriction analysis and by double-strand DNA sequencing (Chen & Seeburg, 1985).

Cell Culture, DNA Transfection, and CAT Assays. ROS 17/2.8 cells were cultured in modified F12 medium containing 10% fetal calf serum. UMR 106 (a gift from Dr. T. J. Martin, University of Melbourne, Melbourne, Australia) and CHO cells were cultured in Dulbecco's modified Eagle's medium containing 1% nonessential amino acids and 10% fetal calf serum. All reagents used for tissue culture were obtained from Gibco Laboratories (Grand Island, NY). DNA transfections were performed by the DEAE-dextran method (Lopata et al., 1984; Yoon et al., 1988). Transfected cells were incubated with and without 10 nM $1,25(OH)_2D_3$ (a gift from Dr. Uskovic, Roche Institute, Nutley, NJ) and harvested 48 h after transfection. The CAT assays were performed according to the method of Gorman et al. (1982). The CAT activity was quantitated by scintillation counting of acetylated products separated on thin-layer chromatography and was calibrated to the total amount of protein in corresponding cell extracts. Protein was measured with Coomassie brilliant blue R (Spector, 1978).

Primer Extension. Primer extension was performed essentially as described by Tsang et al. (1982). In a sealed capillary tube, approximately 10 ng of the ^{32}P-end-labeled 45-mer oligonucleotide described above was mixed with 50 μg of rat calvarial total RNA in 10 μL of buffer containing 0.4 M NaCl and 10 mM Pipes, pH 6.4. The mixture was heated to 80 °C for 10 min and incubated at 60 °C overnight. The annealed primer was extended with AMV reverse transcriptase for 45 min at 42 °C, and the products were separated through a 6% polyacrylamide/7 M urea gel. Autoradiography of the dried gel was performed with Kodak XAR film.

Northern and Southern Blot Analysis. Total RNA from rat tissues was prepared as described by Chirgwin et al. (1979), fractionated in a 0.8% agarose gel containing 0.44 M formaldehyde, and transferred to Hybond N (Amersham, Arlington Heights, IL). The 1.6-kb *Pst*I fragment of the osteocalcin gene was labeled by the method of Feinberg and Vogelstein (1984) and was used as a probe for hybridization. The Northern blot was hybridized at 42 °C in a solution containing 50% formamide/6 × SSC/5 × Denhardts/0.1% SDS/salmon sperm DNA (200 μg/mL) and 1 × 10^6 cpm/mL of probe. The Southern blot was hybridized at 65 °C in a solution similar to that used in Northern blot hybridization, but without formamide. The filter was washed in 0.1 × SSC/0.1% SDS at 65 °C, and the film was exposed for 16 h with two intensifying screens.

Results

Isolation and Characterization of the Rat Osteocalcin Gene. Three recombinant clones were isolated after 10^6 phages were screened from a rat genomic library with the 45-mer oligonucleotide described under Materials and Methods. These clones were shown to be identical by restriction analysis and contained two *Eco*RI rat genomic DNA inserts of 7.9 and 4.0 kb. The 7.9-kb *Eco*RI fragment, which hybridized strongly to the 45-mer, was analyzed by restriction digestion and DNA sequencing and was shown to contain four exons that coded for the entire osteocalcin cDNA (Figure 1A). Figure 1B shows the DNA sequence of approximately 2 kb of the osteocalcin gene. The amino acid sequences coded by the four exons are shown under the DNA sequence. Immediately upstream from the initiation codon is ACACC, a sequence strongly homologous to the consensus sequence suggested to be involved in the initiation of translation of eukaryotic mRNA (Kozak, 1984). The sequences at the 5′ and 3′ ends of each intron correspond to the consensus sequence (AG/GT) of intron–exon boundaries in eukaryotic genes (Green, 1986). The poly(A) tail of rat osteocalcin cDNA (Pan & Price, 1985) is added 18 bases downstream of the consensus sequence AATAAA. The common G/T-rich region for the processing of the 3′ end of eukaryotic mRNA is also present downstream of the poly(A) addition site (Birnstiel et al., 1985).

The rat osteocalcin gene has 81% sequence homology with the human gene in the coding region and 72% homology overall (Celeste et al., 1986). The first three exons in the human and rat osteocalcin genes are of identical size, 64, 33, and 70 bp, respectively. The last exon of the rat osteocalcin gene is longer by three bases encoding an additional amino acid, threonine (Celeste et al., 1986). In contrast to the strong conservation of the exon sequences, intron size and sequences vary considerably between the human and rat osteocalcin genes. However, substantial homology extends into the 5′-flanking region, indicating functional importance for these sequences.

Southern blot analysis showed that a 7.9-kb *Eco*RI and a 1.6-kb *Pst*I rat genomic fragment hybridized to the 1.6-kb *Pst*I probe prepared from the isolated genomic clone (Figure 2). This simple hybridization pattern was identical with the restriction pattern of the isolated genomic clone, indicating that osteocalcin is a single gene product.

The 5′-Flanking Region of the Rat Osteocalcin Gene. The 5′ end of the osteocalcin mRNA was mapped by primer extension as described under Materials and Methods. An extension product five bases longer than the primer (Figure 3, lane 2) locates the site of transcription initiation at the position designated as +1 in Figure 1B. Similar to other eukaryotic promoters, the "TATA" sequence (TATAAAA) is located between bases −31 and −25 and the "CAT" sequence (CCAAT) was located between bases −92 and −88. The promoter region is not G/C rich and does not contain a consensus sequence for the SP1 binding site (Briggs et al., 1986). By computer analysis, several other putative cis-acting sequences involved in the regulation of transcription were identified, including AP1, AP2, and NF1 binding domains, an enhancer corelike sequence, and a cAMP-responsive region (Figure 1B; Mitchell et al., 1987; Jones et al., 1987; Weiher et al., 1983; Maurer et al., 1987; Comb et al., 1986).

Tissue Specificity of Osteocalcin Expression. The tissue distribution of osteocalcin mRNA was investigated in ten rat tissues by Northern blot analysis, using the 1.6-kb *Pst*I genomic fragment as a hybridization probe. The probe hybridized to a single 0.6-kb mRNA only in RNAs isolated from rat calvariae and ROS 17/2.8 cells (Figure 4). None of the other tissues examined, including brain, heart, intestine, kidney, liver, lung, skin, spleen, and testis, showed hybridization, even with longer film exposures. This finding indicates that osteocalcin expression is bone specific among the tissues surveyed.

Promoter Activity and Its Regulation by $1,25(OH)_2D_3$. To examine the promoter activity of the 5′-flanking sequences,

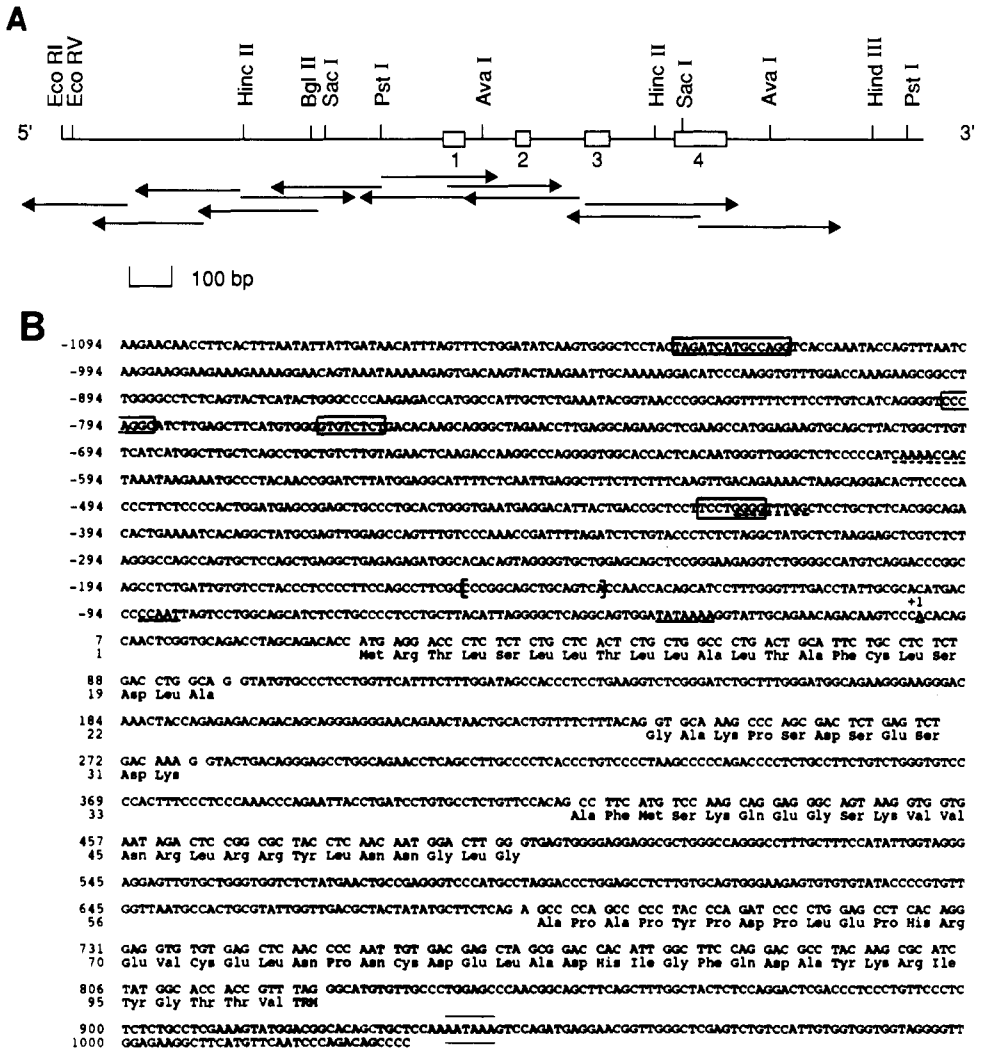

FIGURE 1: (A) Structure of the rat osteocalcin gene. The rat osteocalcin gene contains four exons indicated by open boxes. The arrows indicate the direction and extent of DNA sequencing performed by the Sanger dideoxy method. Restriction sites are shown at the top. (B) DNA sequence of the rat osteocalcin gene. Coding sequences are shown together with the translated amino acid sequence. The initiation of transcription is marked as +1 at residue A. The TATA and CCAAT box sequences are underlined. The polyadenylation signal sequence (AATAAA) is over- and underlined. DNA sequences closely corresponding to several consensus sequences are indicated as boxes. These elements are putative DNA binding sequences for the following: NF1, 5′-TAGATCATGCCAGG-3′ at −1029 to −1016; AP2, 5′-CCCAGGC-3′ at −797 to −791 and 5′-TCCTGGGG-3′ at −426 to −419 (reverse orientation); AP1, 5′-GTGTCTCT-3′ at −771 to −764. Two enhancer corelike sequences are indicated as a dotted line at −422 to −414 and at −603 to −595 (reverse orientation). A possible cAMP-responsive element is shown in brackets at −154 to −138.

a 1.2-kb fragment containing bases −1094 to +147 of the osteocalcin gene was inserted upstream of the CAT gene. Both CAT plasmids containing 5′–3′ and 3′–5′ orientations were constructed and transfected into ROS 17/2.8 cells. Extracts from cells transfected with the plasmid containing the 5′–3′ orientation (pOCCAT) showed detectable CAT activity, which was stimulated 10-fold by 10 nM $1,25(OH)_2D_3$ (Figure 5B, plasmid A). On the other hand, the plasmid containing the 3′–5′ orientation showed no detectable CAT activity (data not shown).

To map the $1,25(OH)_2D_3$-responsive region within the 5′-flanking region of the osteocalcin gene, plasmids containing progressive deletions of the 5′-flanking region were constructed by using the *Exo*III–mung bean nuclease deletion method (Morris et al., 1986). Figure 5 illustrates the size of the deletion and the relative CAT activity measured for each plasmid. Plasmids A (−1094 to +147) and B (−1035 to +147) exhibited similar CAT activity, which was stimulated 5–10-fold by $1,25(OH)_2D_3$ (10 nM). When an additional 13 bp were deleted in plasmid C (−1022 to +147), the CAT activity was reduced to 70% of that exhibited by plasmids A and B, but was still stimulated 9-fold by $1,25(OH)_2D_3$. Plasmids D (−871 to +147) and E (−829 to +147) showed a dramatic decrease in CAT activity, but a weak stimulation by $1,25(OH)_2D_3$ was still detectable (Figure 5C, lanes 7–10). When the 5′-flanking region was deleted to base −335 (plasmid F), no stimulation by $1,25(OH)_2D_3$ was observed (Figure 5C, lanes 11 and 12). This finding indicates that the region located between bases −1035 and −871 is strongly responsive to $1,25(OH)_2D_3$ and that the region located between bases −829 and −335 is weakly responsive to $1,25(OH)_2D_3$. The CAT activities of cell extracts transfected with pSV2CAT did not change in response to $1,25(OH)_2D_3$ (Figure 5C, lanes 13 and 14).

To investigate whether $1,25(OH)_2D_3$ stimulation is dependent on endogenous osteocalcin expression, plasmids A–F were transfected into UMR 106 cells. These cells contain $1,25(OH)_2D_3$ receptors (Partridge et al., 1980) but lack osteocalcin expression (Fraser et al., 1988). No detectable CAT activity was observed in UMR 106 cells transfected with any of these constructs, either in the presence or absence of $1,25(OH)_2D_3$ (Figure 5D). On the other hand, CAT activity was detectable in transfected ROS 17/2.8 cells even in the

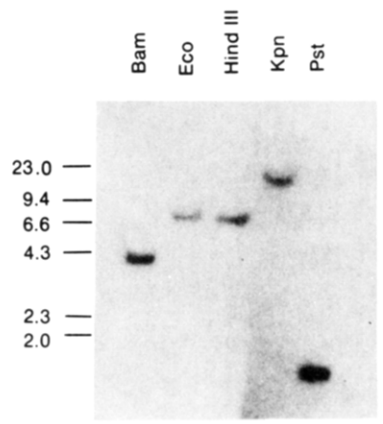

FIGURE 2: Southern blot analysis of rat genomic DNA. Ten micrograms of rat genomic DNA (Clontech, Palo Alto, CA) was digested with the restriction enzymes shown (Bam, *Bam*HI; Eco, *Eco*RI; Hind III, *Hin*dIII; Kpn, *Kpn*I; Pst, *Pst*I). Hybridization was carried out with the ^{32}P-labeled 1.6-kb *Pst*I fragment described under Materials and Methods. Molecular size markers (kb) are *Hin*dIII-digested λ DNA.

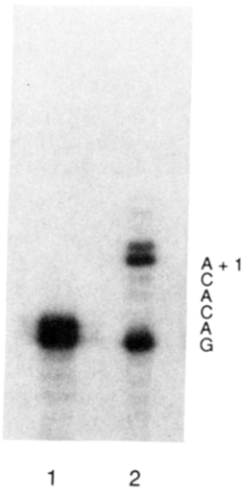

FIGURE 3: Mapping of the 5′ end of the rat osteocalcin mRNA. Radiolabeled primer was annealed to 50 μg of total rat neonatal calvarial RNA and extended with AMV reverse transcriptase as described under Materials and Methods. The primer is seen in lane 1, while the extension products are seen in lane 2. The size of the extension product was determined by a DNA sequencing gel, and corresponding sequences in the osteocalcin gene are indicated in the right lane.

absence of $1,25(OH)_2D_3$ and did not differ appreciably among all constructs within experimental variation (Figure 5B). The transfection efficiency in UMR 106 cells was similar to that of ROS 17/2.8 cells, estimated by use of pSV2CAT plasmids as control (Figure 5C,D, lanes 13 and 14). These plasmids were also transfected to CHO fibroblast cells, which do not express osteocalcin. Similar to the results obtained with UMR cells, no detectable CAT activity was observed in CHO cell extracts (data not shown).

Discussion

A rat genomic fragment containing the osteocalcin gene was characterized, including approximately 1 kb of the 5′-flanking region. Sequence analysis showed that the osteocalcin gene is highly conserved between rat and human. Genomic Southern analysis indicated that osteocalcin is the product of a single-copy gene. Northern analysis of various rat tissues detected osteocalcin mRNA only in bone, a finding consistent with previous observation (Price, 1983). The 5′-flanking region

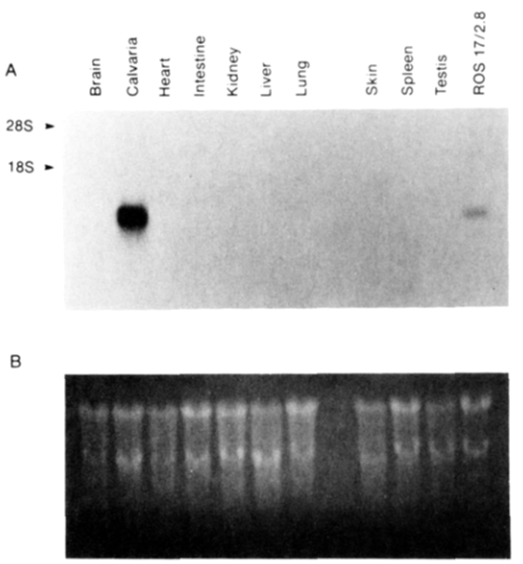

FIGURE 4: Tissue distribution of osteocalcin mRNA. (A) Total RNAs (20 μg) from various tissues were separated by electrophoresis and transferred to a nylon membrane. Hybridization was carried out with the ^{32}P-labeled 1.6-kb *Pst*I fragment. The size of the rat osteocalcin mRNA was previously determined to be approximately 0.6 kb (Pan & Price, 1985). The positions of 28S and 18S ribosomal RNA are indicated. (B) The equal intensity of ethidium bromide fluorescence in positions corresponding to the ribosomal RNAs indicates that an equal amount of RNA was loaded in each lane.

contained several cis-regulatory sequences, including a NF1 binding domain and a cAMP-responsive region. The loss of the putative NF1 domain by the deletion of 13 bp in plasmid C (Figure 5A) could account for the 30% decrease in CAT activity observed both in the presence and in the absence of $1,25(OH)_2D_3$ (Figure 5B). The sequence of the cAMP-responsive element is highly conserved in the human and rat genes and is located at approximately the same position. It was recently shown that adenylate cyclase stimulating agents, such as parathyroid hormone, cholera toxin, and forskolin, increased the steady-state level of osteocalcin mRNA (Noda et al., 1988; Theofan & Price, 1988).

Deletion analysis of the 5′-flanking region of the osteocalcin gene identified a domain strongly responsive to $1,25(OH)_2D_3$, located between bases −1035 and −871. A weaker responsive region was located further downstream. Comparison of these regions with the DNA sequences of two other $1,25(OH)_2D_3$-inducible genes, rat 8K (Perret et al., 1988) and chicken 28K calbindins (Minghetti et al., 1988), revealed the following sequence similarity:

rat osteocalcin	(-989)	AGGAAGAAAGAAAAGGAACAGT	(-968)
28K chicken calbindin	(-799)	AGGA GAAAGAAA GGCAGGTT	(-780)
8K rat calbindin	(-214)	AGGA GAA GAAAAGCCAGGTT	(-195)

It is of interest that the 5′-flanking region of the chicken 28K calbindin gene containing bases −451 to +50 but lacking the above sequence (−799 to −780) was not responsive to $1,25(OH)_2D_3$ (Ferrari et al., 1988). However, experimental evidence, such as DNA footprinting, will be necessary to establish the relevance of these DNA sequence matches.

The cell-specific expression of osteocalcin was investigated by transfection of plasmids into two osteoblastic cell lines (ROS 17/2.8 and UMR 106) and CHO fibroblast. Fraser et al. (1988), using radioimmunoassay and Northern blot analysis, reported that $1,25(OH)_2D_3$ stimulated osteocalcin expression in ROS 17/2.8 cells but failed to induce its expression in UMR 106 cells. In the absence of $1,25(OH)_2D_3$,

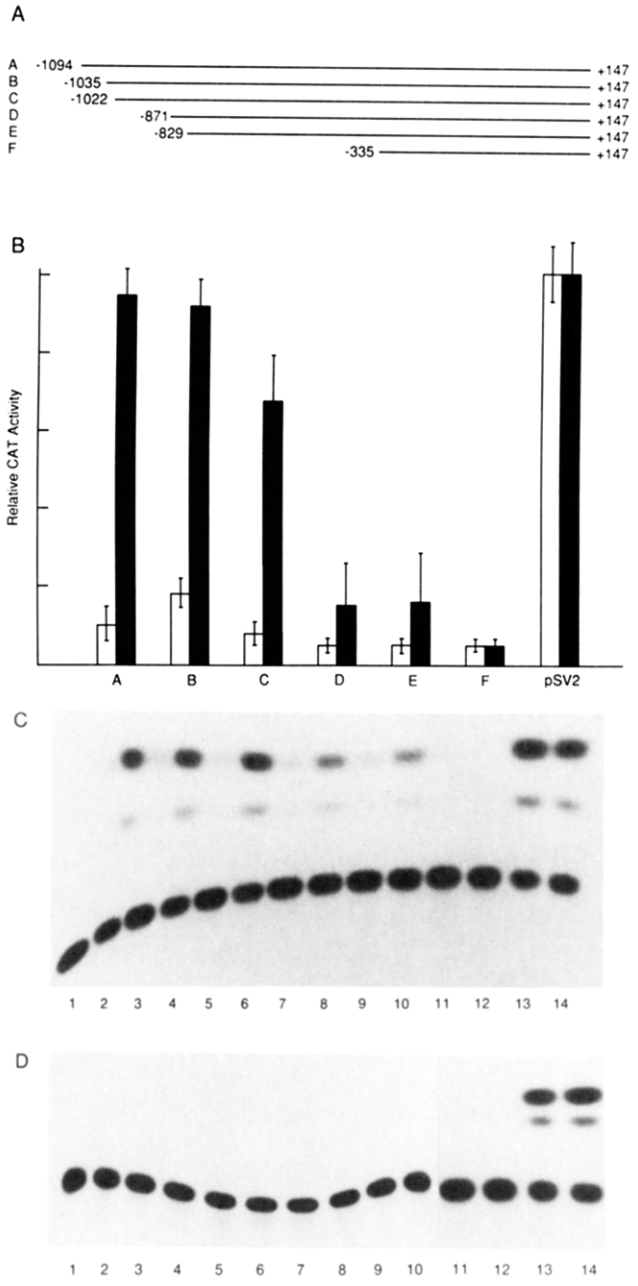

FIGURE 5: (A) Diagram of deletions in the osteocalcin promoter–CAT constructs. The 5′-flanking region of the osteocalcin gene was deleted progressively at the 5′ end by the *Exo*III–mung bean nuclease method (Morris et al., 1986). Numbers indicate the position of the deletion. (B) $1,25(OH)_2D_3$ regulation of the CAT activity in transfected ROS 17/2.8 cells. Plasmids A–F and pSV2CAT were transfected into ROS 17/2.8 cells by the DEAE-dextran method. Relative CAT activities were normalized to that of pSV2CAT as a control and plotted in the absence (open box) and presence (filled box) of 10 nM $1,25(OH)_2D_3$. Each value represents the mean of the CAT activities measured in five separate transfection experiments with the standard deviation indicated by bars. (C) Autoradiogram corresponding to one of the experiments included in (B). Lanes 1, 3, 5, 7, 9, 11, and 13 correspond to the CAT activities exhibited by the cell extracts of ROS 17/2.8 cells transfected with plasmids A–F and pSV2CAT, respectively, in the absence of $1,25(OH)_2D_3$. Lanes with even numbers correspond to the CAT activities with plasmids A–F and pSV2CAT, respectively, in the presence of 10 nM $1,25(OH)_2D_3$. The lower band corresponds to unacetylated chloramphenicol (Cm), and the two higher bands indicate acetylated Cm. (D) Autoradiogram of the CAT activity exhibited by cell extracts of transfected UMR 106 cells. Lanes are numbered in the same manner as in (C).

transfected ROS 17/2.8 cells exhibited a higher CAT activity (Figure 5C) compared to transfected UMR 106 (Figure 5D) or CHO cells (data not shown). Moreover, $1,25(OH)_2D_3$ stimulated CAT expression only in ROS 17/2.8 cells and not in UMR 106 cells. Thus, CAT expression in transfected ROS 17/2.8 and UMR 106 cells is consistent with the reported endogenous expression of osteocalcin in these cell lines, suggesting the involvement of a putative cell- or differentiation-specific factor. It is also possible that UMR 106 or CHO cells express inhibitory factors for the osteocalcin expression. Further studies are under way to identify the molecular basis for this cell-specific regulation.

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Articles

Metabolism of D-Glucose in a Wall-less Mutant of *Neurospora crassa* Examined by ^{13}C and ^{31}P Nuclear Magnetic Resonances: Effects of Insulin†

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ABSTRACT: ^{13}C NMR and ^{31}P NMR have been used to investigate the metabolism of glucose by a wall-less strain of *Neurospora crassa* (slime), grown in a supplemented nutritionally defined medium and harvested in the early stationary stage of growth. With D-[1-^{13}C]- or D-[6-^{13}C]glucose as substrates, the major metabolic products identified from ^{13}C NMR spectra were [2-^{13}C]ethanol, [3-^{13}C]alanine, and C_1- and C_6-labeled trehalose. Several observations suggested the existence of a substantial hexose monophosphate (HMP) shunt: (i) a 70% greater yield of ethanol from C_6- than from C_1-labeled glucose; (ii) C_1-labeled glucose yielded 19% C_6-labeled trehalose, while C_6-labeled glucose yielded only 4% C_1-labeled trehalose; (iii) a substantial transfer of ^{13}C from C_2-labeled glucose to the C_2-position of ethanol. ^{31}P NMR spectra showed millimolar levels of intracellular inorganic phosphate (P_i), phosphodiesters, and diphosphates including sugar diphosphates and polyphosphate. Addition of glucose resulted in a decrease in cytoplasmic P_i and an increase in sugar monophosphates, which continued for at least 30 min. Phosphate resonances corresponding to metabolic intermediates of both the glycolytic and HMP pathways were identified in cell extracts. Addition of insulin (100 nM) with the glucose had the following effects relative to glucose alone: (i) a 24% increase ($P < 0.01$) in the rate of ethanol production; (ii) a 38% increase ($P < 0.05$) in the rate of alanine production; (iii) a 27% increase ($P < 0.05$) in the rate of glucose disappearance. Insulin thus increases the rates of production of ethanol and alanine in these cells, in addition to increasing production of CO_2 and glycogen, as previously shown.

Recently, the wall-less "slime" mutant of *Neurospora crassa* has been shown to respond to mammalian insulins. McKenzie et al. (1988) demonstrated that addition of modest concentrations (10-100 nM) of bovine, porcine, or human insulin to these cells, under defined conditions, resulted in growth stimulation and prolonged viability in a dose-dependent fashion. Treatment with insulin for as little as 30 min led to a 20% increase in the rate of carbon dioxide production from D-glucose (McKenzie et al., 1988), increased the cellular glycogen content, increased the rate of the enzyme glycogen synthesis, and caused specific activation of glycogen synthetase

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