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Construction of DNA Sequences Complementary to Rat α_1 and α_2 Collagen mRNA and Their Use in Studying the Regulation of Type I Collagen Synthesis by 1,25-Dihydroxyvitamin D†

C. Genovese, D. Rowe,* and B. Kream

ABSTRACT: Type I collagen mRNA from fetal rat calvaria was used as a template for the synthesis of a cDNA that was subsequently inserted in the *Pst*I site of the plasmic vector pBR322 and cloned. Three recombinant plasmids containing type I collagen specific sequences were characterized: pα_1R1 is 1600 bp and spans approximately 500 amino acid residues within the triple helical region of α_1(I) and pα_1R2 is 900 bp in size and covers the entire 3′ noncoding and about half of the C-terminal propeptide region of α_1(I) collagen mRNA. The third recombinant pα_2R2 is 1500 bp and contains α_2(I) sequences specific for the entire 3′ noncoding and C-terminal propeptide region. Partial nucleic acid sequence data revealed that the decreasing order of amino acid and nucleotide homology to similar regions of the rat cDNA was mouse > human > chick. Northern hybridization of mRNA after electrophoresis in 0.8% agarose revealed two distinctly different molecular weight patterns characteristic of α_1(I) (4.7 and 5.7 kb) and α_2(I) (4.2 and 4.5 kb) collagen mRNA when hybridized with the corresponding cDNA probe. Despite the high degree of sequence homology, DNA probes from rat or human produced a significantly reduced hybridization signal when used as an interspecies hybridization probe than to its corresponding mRNA. The rat cDNA probes were used in a dot hybridization assay to measure the type I collagen mRNA content in the fetal rat calvaria. The 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] reduced collagen synthesis and type I collagen mRNA levels in osteoblasts located in the central bone segment of calvaria but had no effect on cells in the periosteum. Furthermore, 1,25-$(OH)_2D_3$ appeared to regulate the levels of the α_1(I) and α_2(I) collagen mRNA in a coordinated manner.

Collagen is the most abundant structural protein in vertebrates. It provides support for body organs and plays an important role in development and cell–cell interactions. Thus, it is not surprising that its production is highly regulated. For example, transcriptional regulation has been observed in the Rous sarcoma transformed chick embryo fibroblasts (Sandmeyer & Bornstein, 1981; Sobel et al., 1981) and during development of chick calvaria (Moen et al., 1979). Translational control is mediated by the cleaved N-terminal propeptide of type I procollagen (Horlein et al., 1981). Posttranslational regulation of the collagen synthesis is well documented at the level of intracellular degradation (Berg et al., 1980). Our laboratory has studied the mechanism(s) by which parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$][1] regulate bone collagen synthesis. Using cell-free translation we have shown that the collagen synthesis and the steady-state mRNA levels are diminished to the same degree in fetal rat calvaria incubated in the presence of these hormones (Kream et al., 1980; Rowe & Kream, 1982). To define further the mechanism of the regulation, cDNA probes to chick and human type I collagen were hybridized to rat mRNA extracted from cultured rat calvaria. Because this heterologous hybridization signal was weak, we developed rat-specific type I collagen cDNA probes. This report describes the synthesis and characterization of these probes. These cDNA probes have sufficient sensitivity to measure changes in type I collagen mRNA in the central bone and periosteal components of fetal rat calvaria. Using a dot hybridization assay, we determined that 1,25-$(OH)_2D_3$ reduced the relative type I collagen mRNA content and collagen synthesis in the central bone but not the periosteum.

Experimental Procedures

Construction and Cloning of cDNA. Calvaria from 40–50, 19-day-old fetal rats were removed, cleaned free of contaminating tissues and surrounding epithelium, and immersed in liquid N_2. The RNA was extracted in SET buffer (1% SDS, 5 mM EDTA, 10 mM Tris, pH 7.5) containing 50 μg/mL proteinase K (Rowe et al., 1978). The 28S RNA was isolated by SDS–sucrose gradient centrifugation, and the mRNA was poly(A) selected by passage through an oligo(dT)-cellulose

† From the Departments of Pediatrics (C.G. and D.R.) and Medicine (B.K.), the University of Connecticut Health Center, Farmington, Connecticut 06032. *Received January 10, 1984; revised manuscript received June 28, 1984.* Supported by NIH Grants AM29983 and AM29850. D.R. is a recipient of RCDA HD00330, and B.K. is a recipient of RCDA AM01017.

[1] Abbreviations: PTH, parathyroid hormone; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethylcellulose; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SSC, 0.15 M NaCl–0.015 M sodium citrate; dGTP, deoxyguanosine triphosphate; dATP, deoxyadenosine triphosphate; dTTP, thymidine triphosphate; dCTP, deoxycytidine triphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TCA, trichloroacetic acid; CDP, collagenase-digestible protein.

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column (Aviv & Leder, 1972). Single-stranded cDNA was synthesized in a 250-μL reaction mixture containing 50 mM Tris, pH 8.3, 8 mM $MgCl_2$, 40 mM KCl, 100 μg/mL actinomycin D (Sigma), 5 μg/mL oligo(dT); 500 μM each of dGTP, dATP, dTTP, 200 μM dCTP, 0.5 mCi/mL [^{32}P]dCTP, and 100 μg/mL mRNA (Wickens et al., 1978; Myers et al., 1977). The mRNA was dissolved in 20 μL of H_2O, adjusted to 2.5 mM CH_3HgOH (Alpha), and incubated at room temperature for 2 min and then at 50 °C for 1 min (Payvar & Schimke, 1979). The RNA was then adjusted to 10 mM dithiothreitol and placed on ice for 1 min prior to the addition of the reaction mixture. Upon addition of the reaction mixture, the sample was heated at 42 °C for 5 min followed by chilling on ice. Avian myeloblastosis virus (AMV) reverse transcriptase (a gift from Dr. James Beard, Life Science, Inc., St. Petersburg, FL) was added to the reaction to a concentration of 150 units/mL and incubated at 42 °C for 1 h. The reaction was terminated by addition of EDTA to 5 mM, and the mRNA was hydrolyzed in 0.3 M NaOH at 65 °C for 15 min.

The second DNA strand was synthesized with the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim), followed by digestion with S-1 nuclease. Tailing of the dscDNA with deoxycytidine and the linearized, alkaline phosphatase-treated pBR322 with deoxyguanidine followed the method of Nelson & Brutlag (1979). Equal picomoles of tailed dscDNA and tailed plasmid were annealed in a 10-μL reaction and introduced into *E. coli* N-38 cells (C600, r^-, m^-) made competent for transformation by using a modification of previous procedures (Ener et al., 1975; Dagert & Ehrlich, 1979; Kushner, 1978). The cells were grown to an OD_{550} of 0.400–0.600 at 37 °C. All subsequent steps were performed at 4 °C. Two-milliliter aliquots of cells were collected in 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, and 10 mM RbCl by centrifugation for 5 min at 1500*g*. The cells were resuspended in 100 μL of 100 mM MOPS, pH 6.5, 10 mM RbCl, and 100 mM $CaCl_2$. Three microliters of Me_2SO was added and the cells were kept on ice for 20 h. Ten microliters of the recombinant plasmid was added to the cells. The cells were heated to 43.5 °C for 30 s, diluted to 2 mL with Luria-Betani medium (LB), and incubated for 1 h at 37 °C. One hundred microliters of the cell suspension was diluted to 3 mL in 0.6% agarose and plated over LB plates containing 12.5 μg/mL tetracycline. All tetracycline-resistant transformant colonies were grown on replicate nitrocellulose filters. DNA was fixed onto the filters as previously described (Grunstein & Hogness, 1975). Plasmids containing collagen sequences were detected by hybridization with cloned cDNAs complementary to human and chick type I collagen mRNA sequences: human α_1, pg α_1 H1, a genomic α_1(I) DNA isolated in this laboratory (Solomone et al., 1984); chick α_1, pcg 54 (Lehrach et al., 1979); chick α_2, pcg 45 (Lehrach et al., 1978). The chick cDNAs were generously supplied by Dr. Helga Boedtker, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA.

Sequencing of ^{32}P end label DNA fragments obtained from the cloned dscDNA employed the procedure of Maxam & Gilbert (1980). After the chemical modifications the reaction

Condition of restriction enzyme digestion followed those given by the supplier. Nick translation of the DNA used the DNA polymerase/DNase enzyme solution obtained from Amersham.

Analysis of Type I Collagen mRNA in Fetal Rat Bone. Timed pregnant rats were obtained from the Charles River Breeding Laboratories, Inc. The frontal and parietal bones were removed from 21-day-old fetal rats, split along the saggital suture, and cultured individually in 35-mm plastic tissue culture wells containing 2 mL of modified BGJ medium (Bingham & Raisz, 1974) prepared with 100 μg/mL ascorbic acid, 1 mM unlabeled proline, and 1 mg/mL bovine serum albumin. 1,25-$(OH)_2D_3$ (the generous gift of Dr. M. Uskokovic, Hoffmann-La Roche) was added to the medium as a solution in absolute ethanol so that the final concentration of ethanol did not exceed 0.1% (v/v). The calvaria were shaked on a rocker platform (Bellco) contained in a humidifier incubator maintained at 37 °C and 5% CO_2. Each calvaria was given fresh medium at 24 h and 50 μg/mL β-aminopropionitrile fumarate (Sigma) at 36 h. At 46 h, calvaria were pulsed with 10 μCi [5-^{3}H]proline (New England Nuclear) for 2 h. Calvaria were removed from culture and rinsed with cold phosphate-buffered saline, pH 7.4. Periosteum was separated from the central bone under a dissecting microscope. Each segment was homogenized individually in 5% trichloroacetic acid (TCA) at 4 °C with a glass/glass homogenizer. Precipitates were collected by centrifugation at 3000*g* × 30 min, washed with 5% TCA and then with 10 mM potassium acetate in absolute ethanol, resuspended in 0.5 M acetic acid, and analyzed for the incorporation of [^{3}H]proline into collagenase-digestible protein (CDP) by using purified bacterial collagenase (Peterkofsky & Diegelmann, 1971). The DNA content was determined by fluorometry using diaminobenzoic acid (Kissane & Robins, 1958).

Identically treated calvaria, used for quantitation of procollagen mRNA levels, were not pulsed with [^{3}H]proline. At the end of culture, these calvaria were rinsed with phosphate-buffered saline and dissected as described above. For each analysis, central bone or periosteum from four half-calvaria were pooled and homogenized with a Polytron in SET buffer containing 50 μg/mL proteinase K. Total RNA was extracted and serially diluted by using 20× SSC (0.15 M NaCl and 0.015 M trisodium citrate) and dotted onto nitrocellulose membrane. All samples contained a minimum of 15× SSC prior to this application onto the membrane. *E. coli* tRNA was added to each dilution such that a total of 2 μg of RNA was dotted. Both the nitrocellulose membrane (HA, Millipore) and an underlying 3MM paper were wetted in water and then soaked in 20× SSC prior to dotting. The filters were baked at 80 °C for 2 h, prehybridized, and hybridized as described by (Thomas, 1980) by using approximately 5 × 10^6 cpm of nick-translated cDNA probes/filter. After hybridization at 42 °C for 12–16 h, the filters were washed in 0.1× SSC/0.1% SDS at room temperature, air-dried, and exposed to Kodak RP X-Omat film for 3 days at −80 °C. The dot intensities were quantitated by densitometry and standard curves were constructed for arbitrary scanner units vs. the dilution of RNA dotted. Slopes of the linear portions of these curves were

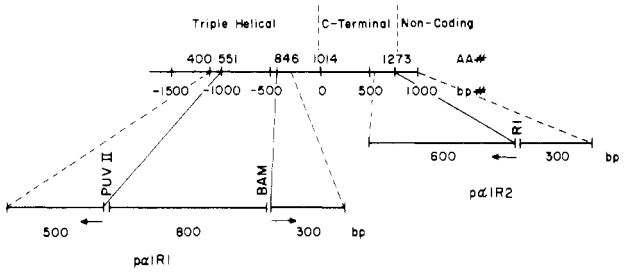


FIGURE 1: Restriction map of the two α_1(I) cDNA clones. pα_1R1 was sequenced from a *Pvu*II and *Bam*HI site as indicated by the arrows. pα_1R2 was sequenced from an *Eco*RI site. Solid lines indicate localization of the cDNA as determined by DNA sequencing while the broken lines show the approximate localization based on the size of the cDNA. Only one strand of DNA was sequenced. The map of the α_1(I) mRNA designates the junction of the helical C-terminal domains as the 0 point while the amino acid residues are counted from the N-terminal telopeptide residue of the collagen α_1(I) chain.

plasmid containing collagen DNA sequences, modifications of the standard methods (Wickens et al., 1978; Myers et al., 1977) had to be employed. Rat calvaria were selected as a source of mRNA since collagen represents approximately 20% of total protein synthesized in this tissue. We found that the collagen mRNA could be purified from contaminating ribosomal RNA to a much higher degree if the total RNA was first size selected on a sucrose gradient followed by enrichment of the 28S RNA for poly(A+) mRNA by oligo(dT) chromatography rather than by the reverse order (data not shown). With the strategy used here, the yield of poly(A+) selected DNA was 0.8% and the ribosomal RNA contamination was minimized.

Prior to selection of the final conditions that were used for constructing the recombinant plasmids, the reverse transcriptase reaction was optimized for the size of the first strand and extent of second strand formation. We selected conditions that resulted in a mean single-stranded size of approximately 2000 bases that extended above 4000 bases. After double standing the size of the cDNA approximately doubled and after S1 nuclease treatment the size fell to a mean of approximately 1500 bases.

E. coli N38 cells treated with the modified calcium/rubidium method gave a transformation efficiency that approached 1×10^7 transformants/μg of DNA. There was no detectable background when cells were transformed with the dG-tailed pBR322 lacking the dC-tailed insert. Recombinants of dC-tailed DNA gel markers annealed with dG-extended pBR322 resulted in a transformational efficiency in the range of (1–10) $\times 10^4$ colonies/μg of DNA.

Characterization of cDNA Clones. Using the recombinant DNA and modified transforming procedure described in the methods, we obtained 186 transformants from 60 ng of DNA of which 11 hybridized with the various probes. Those colonies containing inserts greater than 1000 bases in size and that hybridized strongly with either the chick and human α_1 or α_2 cDNA probes were selected for a further study. Three isolates, two that eventually proved to be α_1 and one α_2 specific, were extensively characterized.

The insert identified as pα_1R1 is 1600 bases long. It codes for residues within the helical region of the α_1 chain estimated to encompass amino acid no. 400–950. Its restriction map is illustrated in Figure 1. Two regions of the cDNA were sequenced. The first is a *Pvu*II/*Pst*I fragment from the 5′ region of the insert that codes for amino acid no. 513–551 in the collagen helix. This amino acid sequence identically

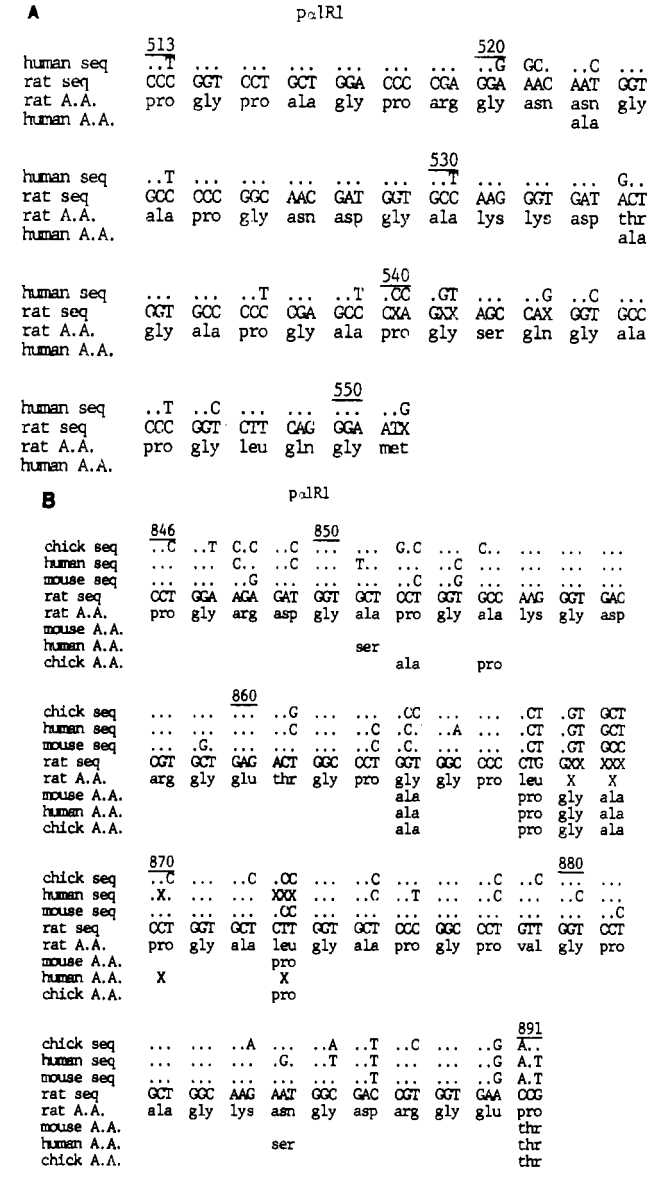


FIGURE 2: Nucleotide sequence data from two regions of pα_1R1. (A) The amino acid sequence 513–551 obtained from the nucleotide sequence is identical with the amino acid using protein sequence data (Fietzek & Kuhn, 1976). (B) The nucleotides corresponding to amino acid residues 846–891 are compared to chick and human nucleotide sequences and the corresponding amino acids. Only nucleotide or amino differences from that obtained from the sequencing of the rat cDNA are shown. Nucleotides marked X represents unreadable bases due to their position on the sequencing gel. Amino acids 864, 867, 868, 869, 873, and 891 were considered ambiguous and not included in the calculation of Table I.

matches that obtained by protein sequencing of rat α_1(I) collagen (Figure 2A). The nucleotide and corresponding amino acid sequence of the human α_1(I) chain (Benard et al., 1983) are also shown. Excluding regions of sequence ambiguity, the homology in this region is 89% for nucleotides and 95% for amino acids. The second region is a *Bam*HI/*Pst*I fragment from the 3′ region of the insert that codes for amino acids 846–891 (Figure 2B). Its nucleotide sequence was compared to the chick (Fuller & Boedtker, 1981), human (Chu et al., 1982; Bernard et al., 1983), and mouse (Monson & McCarthy, 1981) nucleotide sequences and the derived amino acid sequence. In this region a spectrum of amino acid and nucleotide sequence homology to the rat sequence is present in the expected order of mouse > human > chick (Table I, column A). There were no amino acid changes in the mouse–rat sequence, and all but one of the nucleotide

Table I: Percentage Mismatch of Mouse, Human, and Chick Amino Acid (AA) and Nucleic Acid (NA) Sequences to Similar Regions of the Rat α_1(I) and α_2(I) Collagen Chain[a]

	(A) α_1(I) helical domain		(B) α_1(I) C-terminal propeptide		(C) α_2(I) C-terminal propeptide	
species	AA	NA	AA	NA	AA	NA
mouse	0.0	6.6				
human	5.1	11.6	2.6	5.3	0	6.2
chick	5.0	15.8	5.3	15.8	6.8	9.6
total sequence no. analyzed	39	112	38	114	62	186

[a] For these calculations ambiguous sequences indicated in the corresponding figure legends were not included. Column A corresponds to Figure 2B, column B to Figure 3, and column C to Figure 5 until the stop codon is reached.

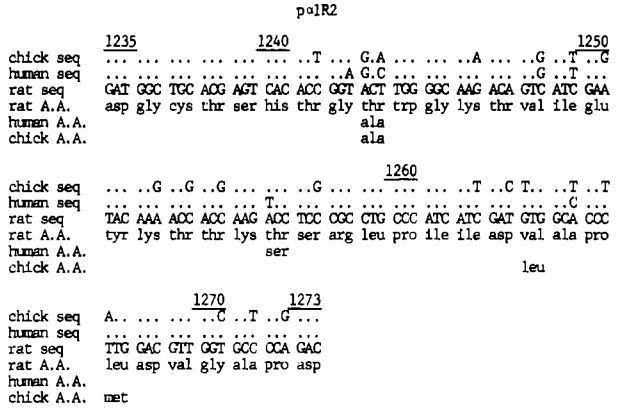
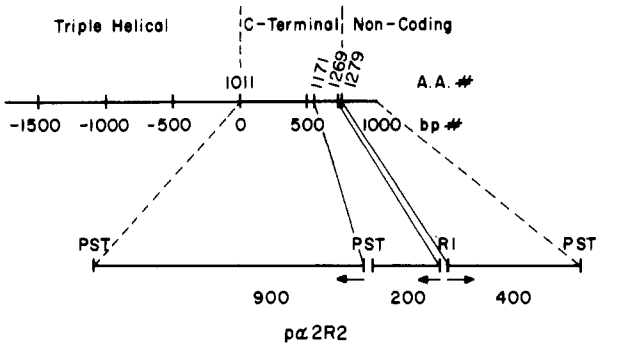


FIGURE 3: Nucleotide sequences from pα_1R2. The nucleotides corresponding to amino acid residues 1235–1273 are compared to those of chick and human. Amino acid residue 1243 was considered ambiguous and omitted from the calculation in Table I.

changes were at the third position.

The second α_1(I) cDNA is designated pα_1R2. It is approximately 900 bp in length and codes for amino acids in the nonhelical region of the α_1(I) chain as illustrated in Figure 1. From an *Eco*RI site within this DNA, a nucleotide sequence was obtained that corresponds to residues 1235–1270 in chick (Figure 3). Within this nonhelical region the sequence homology to chick is as high and to human higher than that found within the helical region (Table I, column B). From the size of this DNA fragment we estimate that is extends from amino acid residue 1150 to the end of the noncoding region of the α_1(I) mRNA.

The α_2-specific cDNA, pα_2R2, is a 1500-bp fragment that is illustrated in Figure 4. Two coding regions of this DNA have been sequenced. One is a sequence from the 3′ side of the 900-bp *Pst*I/*Pst*I fragment that corresponds to amino acid residues 1151–1171 (Figure 5A). This region shows even greater homology to the corresponding chick and human nucleotide sequence (Table I, column C) since it includes the highly conserved glycosylation site from amino acid residues 1152–1165 (Showalter et al., 1980). Another sequence at the 3′ end of the internal *Pst*I/*Eco*RI fragment correspond to residues 1233–1269 (Figure 5B). The amino acid changes that are observed in this region are somewhat less conservative than those seen in the same region of the α_1 chain. For example, contrasting the rat and chick sequence, there is a substitution of threonine for an asparagine residue at position 1238, a glutamic acid for a lysine residue at position 1240, and an asparatic acid for a glycine residue at position 1242. The rat–human changes are similar at two of the three nonconservative sites. Half of the conservative amino acid substitution of the rat sequence follow the chick pattern while the other



α2 RAT cDNA

FIGURE 4: Restriction map of pα_2R2. See Figure 1 for a legend of this map.

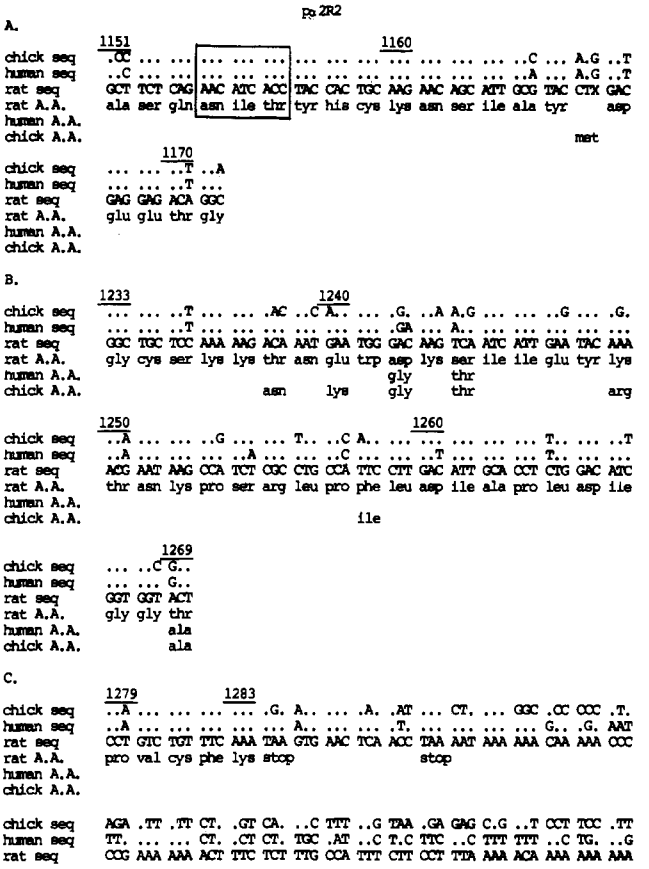


FIGURE 5: Three regions of nucleotide sequence from pα_2R2. Nucleotides corresponding to amino acid residues 1151–1171 (A), 1233–1269 (B), and 1279 to the translation stop site and nontranslated region (C) are compared to similar sequences of chick and human α_2(I) mRNA. The site of the heterosaccharide unit located in sequence A is boxed. Amino acid residues 1166, 1242, 1244, and 1269 were considered ambiguous and were omitted from the calculation in Table I.

half is similar to that of the human. A third sequence was obtained at the junction of the coding and noncoding region from the 5′ end of the 400-bp *Eco*RI/*Pst*I fragment (Figure 5C). Once the noncoding region of the message is reached at residue 1283, the homology of the nucleotide sequence between chick, human, and rat diverges widely. Thus, the overall length of pα_2R2 was estimated to include the entire C-terminal propeptide and to span the full length of the RNA through the noncoding region.

Despite the high homology at the nucleotide level between the chick, human, and the rat cDNA sequences, we found a weaker signal when cross species hybridization was performed.

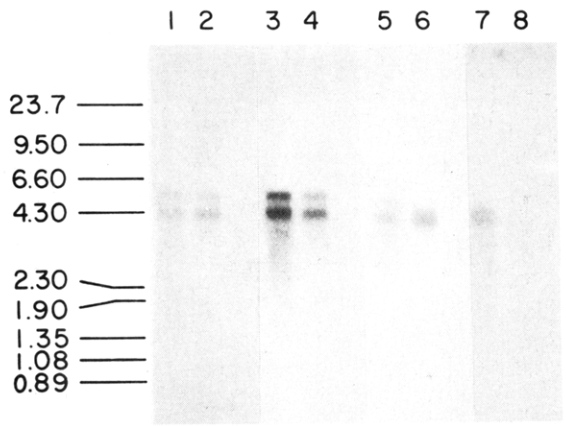


FIGURE 6: Hybridization of rat and human α_1(I) and α_2(I) DNA probes to RNA from rat and human. RNA obtained from human fibroblasts culture or fetal rat calvaria were purified to the point of sucrose gradient ultracentrifugation (see Experimental Procedures). One microgram was electrophoresed in 0.8% agarose containing 1.1 M formaldehyde (Lehrach, 1977) and transferred to nitrocellulose (Thomas, 1980). The matrix-bound RNA was hybridized sequentially with four separate probes. The previously bound DNA probe was removed by washing the filter 2 × 10 min in 50% formamide/2× SSC at 65 °C and rinsed in 2× SSC. Each lane pair has the rat RNA on the left side and the human RNA on the right. Each lane pair was hybridized with a separate DNA probe: 1 and 2, pg α_1H-1, human α_1(I); 3 and 4, pα_1R1 rat, α_1(I); 5 and 6, HpCl, human α_2(I); 7 and 8, pα_2R2, rat α_2(I).

Figure 6 shows a formaldehyde/0.8% agarose gel electrophoresis of human (lanes 2, 4, 6, 8) and rat (lanes 1, 3, 5, 7) 28S RNA that is hybridized with a cDNA for either human or rat α_1(I) or α_2(I) coding sequences. As noted by others (Chu et al., 1982; Adams et al., 1972), the α_1(I) and α_2(I) mRNA bands migrate as distinctly different molecular weight species. In our gel system the strongest α_1(I) band is located at 4.7 kb (lanes 1–4) while the weaker band is found at 5.7 kb. The α_2(I) mRNA species resolve less distinctly, having migration of 4.5 and 4.8 kb (lanes 5–8). The cross hybridization experiments used human genomic sequences. The α_1 probe is a 12-kb fragment containing exons from amino acid 142 in the collagen helix to residue 1271 in the C-terminal propeptide (D. W. Rowe, C. Genovese, M. L. Stover, and M. Poirier, unpublished results). The α_2 probe is a subcloned 1.5-kb *Eco*RI fragment from HpCl that contains the C-terminal propeptide coding sequences (Dalgleish et al., 1982). The rat cDNA pα_1R1 gave a stronger signal to rat RNA than to an equivalent amount of human RNA (lanes 3–4: rat–human RNA probed with rat DNA). When the same RNA was probed with the human α_1 probe, a stronger signal was produced by the human RNA (lanes 1–2: rat–human probed with human DNA). A difference in the hybridization signal was also found by using the rat α_2 probe, pα_2R2 (lanes 7–8: rat–human probed with rat DNA). When the human DNA probe was hybridized to rat RNA, a weak signal was obtained that did not correspond to the expected α_2 collagen pattern (lanes 5–6: rat–human probed with human RNA). The difference in the hybridization signal of rat RNA when probed with chick cDNA probe was greater than when probed with the human DNA probes (data not shown).

Type I Collagen mRNA in Fetal Rat Calvaria. Previous work has indicated that the osteoblast-rich central bone segment of the fetal rat calvaria synthesizes 2–3-fold more collagen than the overlying periosteum (Rowe & Kream, 1982). Furthermore, 1,25-$(OH)_2D_3$ decreased the synthesis of type I collagen only in the osteoblastic-rich central bone. The cDNA probes provide an opportunity to determine if these

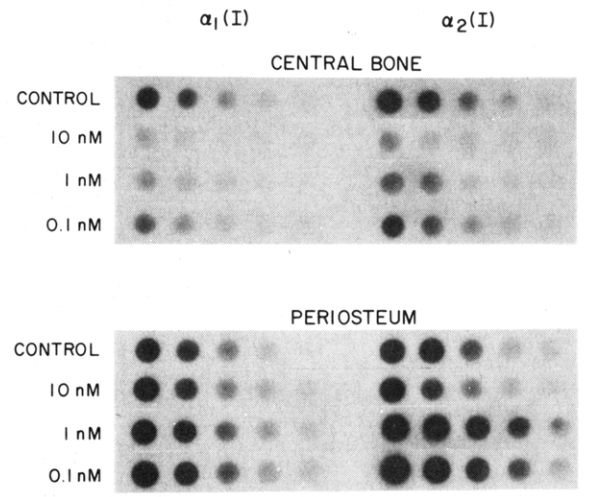


FIGURE 7: Dot blot hybridization of RNA extracted from segments of 21-day fetal rat calvaria treated with 1,25-$(OH)_2D_3$. Fetal rat calvaria were cultured for 48 h in the presence or absence of 0.1–10 nM 1,25-$(OH)_2D_3$. At the end of culture, calvaria were dissected into periosteum and central bone segments. RNA was extracted from pools of four periosteum or central bone segments, immobilized on nitrocellulose filters, and hybridized to ^{32}P-labeled procollagen cDNA probes as described in the text.

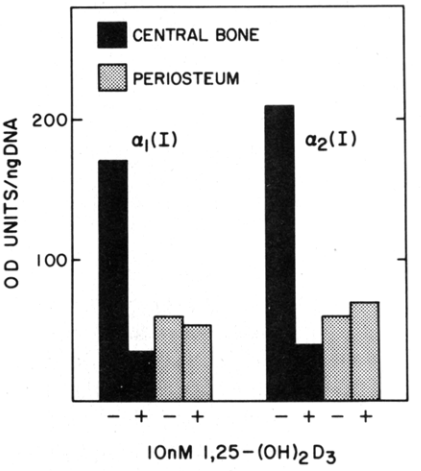


FIGURE 8: Relative content of α_1(I) and α_2(I) collagen mRNA in segments of fetal rat calvaria. Calvaria were incubated in the presence or absence of 10 nM 1,25-$(OH)_2D_3$. Calvaria were dissected into central bone and periosteum. RNA was extracted from pools of four segments, immobilized on nitrocellulose, and hybridized to cDNA probes. The data reflect the slope of the dot hybridization dilution curve divided by a slope of a standard collagen RNA solution. This value is then divided by the DNA content of the sample.

differences are mediated by the type I collagen mRNA content. Figure 7 shows the dot hybridization that was obtained when RNA extracted from each tissue compartment was hybridized with the pα_1R1 and pα_2R2. Although the periosteum produces a stronger hybridization signal, the cell content of the tissue is 2–3 times greater than that of the central bone region. The relative content of type I collagen in each tissue compartment and its responsiveness to 1,25-$(OH)_2D_3$ are illustrated in Figure 8. The data are calculated as OD units of the sample relative to the OD units of a standard RNA and are divided by the DNA content of the sample. When the data are expressed in this manner, the central bone has 2–3-fold more type I collagen mRNA than the periosteum. When treated with 1,25-$(OH)_2D_3$, the collagen mRNA content in the central bone falls to the same level observed in the periosteum. The reduction in type I collagen mRNA was accompanied by a similar fall in collagen synthesis

Table II: Effect of 1,25-Dihydroxyvitamin D_3 on Collagen Synthesis and Procollagen mRNA Levels in Segments of Fetal Rat Calvaria[a]

		treated/control		
	concn (nM)	% collagen synthesis	α_1 (I) mRNA	α_2 (I) mRNA
central bone				
control		1.00	1.00	1.00
1,25-$(OH)_2D_3$	10	0.41	0.21	0.34
	1	0.47	0.26	0.51
	0.1	0.69	0.42	0.56
periosteum				
control		1.00	1.00	1.00
1,25-$(OH)_2D_3$	10	0.80	1.10	0.80
	1	1.00	0.85	1.20
	0.1	0.98	0.95	1.20

[a] Fetal rat calvaria were incubated in varying concentrations of 1,25-$(OH)_2D_3$ and dissected into periosteum and central bone segments at the end of culture. Each segment was analyzed for α_1(I) mRNA, α_2(I) mRNA, and collagen synthesis as described in the text. The data are expressed as the ratio of the treated divided by the control. Five tissue segments were analyzed for collagen synthesis while two groups containing four segments were extracted for dot hybridization.

and was dose related over a 100-fold range in 1,25-$(OH)_2D_3$ concentration. However, 1,25-$(OH)_2D_3$ did not alter the mRNA content or collagen synthesis in the periosteal cells (Table II). In the central bone it appeared that the levels of α_1(I) and α_2(I) mRNA were modulated in a coordinate manner.

Discussion

Rat calvaria are a useful model for studying the hormonal regulation of bone collagen synthesis (Raisz & Kream, 1983). Recombinant DNA probes to study both transcriptional and translational regulations in this system will be of great value. However, it is important that these probes are chain and collagen-type specific when examining the regulation of either the α_1 or α_2 chains and specific collagen types. Since existing probes for collagen mRNA from other species did not have this degree of sensitivity and discrimination for rat collagen mRNA, we undertook to clone rat-specific sequences.

RNA was extracted by using published methods (Rowe et al., 1978; Aviv & Leder, 1972) and purified by sucrose gradient centrifugation followed by oligb(dT) chromatography. Instead of obtaining a yield of approximately 5–10% of total RNA after oligo(dT) chromatography as is usual, the yield was 0.8% when total RNA was first size selected. This percentage is close to the yield expected if the population of mRNA eluted from the oligo(dT) were not contaminated with ribosomal RNA and if the majority of smaller mRNA species were removed.

Conditions were optimized for cDNA transcription from the mRNA and formation of double-stranded cDNA. These steps were monitored by measuring the size of the product and its S1 nuclease sensitivity. We found that the inclusion of vanadyl ribonucleoside complex (Berger & Birkenmeirem, 1979), the elimination of sodium pyrophosphate (Myers & Spiegelman, 1978), and the initial denaturation of the RNA in methylmercury hydroxide (Payvar & Schimke, 1979) increased the size of the cDNA and the transcriptional efficiency. Inclusion of actinomycin D was essential to prevent the formation of hairpin structures (Fagan et al., 1980).

The recombinant DNA probes that have been characterized have sequences that are highly homologous with the nucleotide and amino acid sequence of α_1(I) or α_2(I) chains of chick and man. In one region where a comparison to rat amino acid sequence was possible, identity was found. In those regions where corresponding nucleotide sequences for chick, human, mouse, and rat could be compared, the homology of the rat–mouse exceeded that of human–rat and chick–rat. At the heterosaccharide attachment site in the pro α_2 C-terminal propeptide region, the amino acid and nucleotide sequences were identical in all species. The hybridization pattern to the α_1(I) and α_2(I) mRNA is similar to those noted for human and chick RNA. The results of the cross species hybridization indicate that even with a high degree of nucleotide homology, the hybridization signal is less than the intraspecies hybridization and at times the hybridization may not be chain specific.

Since the hybridization of the rat cDNA probes is sufficiently strong and specific for the α_1(I) and α_2(I) mRNA of type I collagen, these recombinant molecules were used to study the regulation of the collagen synthesis in the fetal rat calvaria. We used a dot hybridization to quantitate the relative content of α_1(I) and α_2(I) mRNA levels in each tissue segment. Our data indicate that the osteoblast-rich central bone contains a higher content of type I collagen mRNA per cell than the periosteum and that cells contained in the central bone respond to 1,25-$(OH)_2D_3$. These data support our previous studies using a cell-free translation assay that showed that 1,25-$(OH)_2D_3$ decreased the activity of procollagen mRNA in calvaria. Furthermore, 1,25-$(OH)_2D_3$ appears to regulate the levels of α_1(I) and α_2(I) mRNA in a coordinated manner. These DNA probes will provide a unique opportunity to study the regulation of the collagen genes in different cell types within the same tissue.

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The Chromoprotein of Halorhodopsin Is the Light-Driven Electrogenic Chloride Pump in *Halobacterium halobium*†

E. Bamberg,* P. Hegemann, and D. Oesterhelt

ABSTRACT: The chromoprotein of halorhodopsin was isolated from *Halobacterium halobium* strain L-33, a bacteriorhodopsin-deficient mutant, and incorporated into asolectin lipid vesicles. When these vesicles are added to one side of a planar lipid membrane, the membrane system becomes photoelectrically active. The observed photoresponse occurs only in the presence of chloride (and other halides). The action spectrum of the photoresponse is identical with the visible absorption band of the chromoprotein in lipid vesicles. The photoresponse consists of a transient photocurrent, which indicates that the lipid vesicles are adsorbed to the surface of the planar lipid membrane but not integrated into it. The stationary photocurrent is extremely low because the underlying lipid membrane is virtually impermeable to the transported ion. The stationary photocurrent, however, increases drastically upon the addition of the lipophilic anion tetraphenyl borate or of the protonophore tetrachloro-2-(trifluoromethyl)benzimidazole (TTFB, HA) to the system. The TTFB-enhanced stationary photocurrent is caused by the transport of an HA_2^- species. The results obtained demonstrate that the chromoprotein of halorhodopsin is the light-driven Cl^- pump in *H. halobium*.

The retinal-binding protein, halorhodopsin, occurs in the cell membrane of *Halobacterium halobium* in addition to the predominant and well-characterized proton pump bacteriorhodopsin (Matsumo-Yagi & Mukohata, 1977; Lanyi & Oesterhelt, 1982; Mukohata & Kaji, 1980; Wagner et al., 1981). On the basis of transport measurements with cell envelope vesicles from the bacteriorhodopsin-deficient mutant strain L-33, it was proposed that halorhodopsin acts as a light-driven chloride pump (Schobert & Lanyi, 1982). This function was demonstrated with cell membrane fragments on black lipid membranes (Bamberg et al., 1984). Both types of experiments, however, do not determine whether the functional unit of halorhodopsin consists of an individual protein species or whether several different proteins contribute to the function. The retinal binding component of halo-

† From the Max-Planck-Institut für Biophysik, D-6000 Frankfurt am Main, West Germany (E.B.), and the Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, West Germany (P.H. and D.O.). *Received March 28, 1984.*

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