Structure of the Promoter of the Rat Type II Procollagen Gene*

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We have isolated several overlapping genomic clones which contain the 5' terminal portion of the rat procollagen (II) chain gene. These clones span about 20 kilobases (kb) of contiguous DNA containing 15 kb of the gene and 5 kb of the 5' flanking sequence. Electron microscopic analysis of mRNA-DNA hybrids by R-looping shows that collectively these clones contain 16 exons which code for approximately one-thirds of the procollagen (II) chain. The sizes of the exons are small, except for the first exon which is relatively large.

The nucleotide sequence of the first exon and the 1000 base pairs (bp) preceding it was determined. The first exon contains a 150-bp untranslated segment and an 85-bp sequence coding for the signal peptide and a part of the NH₂-terminal propeptide of type II collagen. The segment preceding the transcription initiation site contains the "TATA" box and several G + C-rich stretches, whereas the CAT box is not evident between -70 and -120. The hexanucleotide sequence 5'-GCGCGG-3' is found in three different places between -200 and the TATA box. The inverted complement sequence of this hexanucleotide, 5'-CCGCCC-3', is located around -220 and -450. The hexanucleotide and its inverted sequence have been found previously in the promoter region of the tk gene of herpes virus. These sequences are known to function in a mutually dependent manner as transcription signals for the tk gene; thus, they may play a role in determining the level of transcription of this cartilage gene. The hexanucleotide, 5'-CCGCCC-3', is also found in the 21-base pair repeats of the SV40 promoter and the promoter region of hydroyxymethylglutaryl-CoA reductase gene. The sequence 5'-GTGGTTAGA-3' located around -280 is identical to the "core" sequence that has been reported as enhancer element in both viral and cellular genes. These unusual structures may be related to the tissue-specific expression of this gene.

The collagens are abundant proteins that constitute the major structural element in extracellular matrices (1). There are a variety of such collagens, and they are encoded by a family of genes that are differentially expressed in different tissues and during development. Type II procollagen is synthesized specifically by chondrocytes, and type II collagen represents the major collagen in cartilage (2). In early development, there is a switch in synthesis of type I procollagen to type II procollagen, as mesenchymal cells differentiate into chondrocytes. Similar differentiation can be induced in culture. However, the phenotype of the chondrocyte is unstable and changes to a fibroblastic phenotype with extended culture (3) or changes more rapidly when the cells are exposed to agents such as BudR and retinoic acid (4-6).

Our laboratory has initiated studies on the regulation of the procollagen (II) chain gene. We have previously isolated a cDNA clone complementary to the 5' end of procollagen (II) chain mRNA (7). Using this cDNA clone as a probe, we have isolated several overlapping genomic clones containing the 5' end of the rat procollagen (II) chain gene. We have identified the transcription start site and determined the nucleotide sequence of the promoter and of the first exon of this gene.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from Bethesda Research Laboratories and New England Biols (Beverly, MA). Polynucleotide kinase and T4 DNA ligase were purchased from P.L. Biochemicals. 32P-labeled ATP and deoxynucleotide as well as enzymes for nick translation were purchased from Amersham Corp. A partial HeI1I rat genomic DNA library cloned into the plasmid vector was a generous gift of Thomas Sargent, National Institutes of Health.

Hybridization Probes—A 600-bp HindIII-BamHI fragment was isolated from the cDNA clone pRco12, coding for the 5' portion of the rat type II collagen mRNA (7). This fragment contains the untranslated region and the sequence coding for the signal peptide, the aminopropeptide, and a part of the telopeptide. This fragment was labeled with 32P-deoxycytidine by nick translation (8).

Screening of the Rat Genomic Library—A library of the rat genomic DNA fragment was screened by using the cDNA fragment described in the preceding paragraph as a hybridization probe. Hybridization was carried out in 6 × SSC (1 × SSC 0.15 M NaCl and 0.015 M sodium citrate), 10 × Denhardt's solution (1 × Denhardt's, 0.02%) each of bovine serum albumin, Ficoll, and polyvinyl pyrrolidone, 0.1% SDS, and 100 μg/ml sonicated salmon sperm DNA at 68 °C for 16 h. The filters were washed twice with 2 × SSC and 0.1% SDS, and then twice with 0.1 × SSC and 0.1% SDS at room temperature.

DNA Preparation and Southern Hybridization—Charon 4A recombinant bacteriophage and plasmids were purified as described (9). Purified DNA was digested by restriction enzymes, electrophoresed on a 0.7% agarose gel, and transferred to a nitrocellulose filter (9). Hybridization was carried out in 50% formamide, 5 × SSC, 10 × Denhardt's, 0.1% SDS, and 100 μg/ml salmon sperm DNA for 16 h at 37 °C. Filters were washed twice in 2 × SSC, 0.1% SDS at room temperature, and then in 0.1 × SSC, 0.1% SDS.

Preparation of RNA and RNA Blot Hybridization—RNA was isolated from the Swarm rat chondrosarcoma by the guanidine-HCl method as described previously (10). Poly(A) RNA was purified by two cycles of oligo(dT)-cellulose chromatography. RNA was separated by electrophoresis on a formaldehyde-agarose gel and transferred to a nitrocellulose filter (9). Hybridization and washing were carried out as described for the Southern hybridization.

DNA Sequencing—The DNA sequence was determined using the Maxam and Gilbert method (11).

S1 Mapping—S1 mapping was carried out according to the method described by Berk and Sharp (12). Five μg of poly(A) RNA from the rat chondrosarcoma was hybridized with the HindIII-SmaI fragment

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1 The abbreviations used are: bp, base pairs; SDS, sodium dodecyl sulfate; kb, kilobase; PIPES, 1,4-piperazine-diethanesulfonic acid.
labeled at the 5' end with [γ-32P]ATP in 80% formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA for 3 h at 52 °C. The reaction mixture was diluted 10-fold in ice-cold S1 buffer (0.25 M NaCl, 0.03 M Na acetate, pH 4.6, 1 mM ZnSO4) containing either 20 units/ml, 100 units/ml, or 500 units/ml of S1 nuclease. The S1 reaction was performed at 37 °C for 30 min. The reaction was terminated by the addition of 1/4 volume of 2.5 M ammonium acetate and 50 mM EDTA. S1-resistant materials were precipitated with isopropanol collected by centrifugation, resuspended in 80% formamide, and heated at 90 °C for 1 min before loading on a 7 M urea, 8% polyacrylamide gel (11).

Primer Extension by Reverse Transcriptase—The HpaII-Smal DNA fragment, labeled at its SmaI 5' end, was used as a primer. This DNA fragment was denatured at 80 °C for 5 min and then hybridized with poly(A) RNA in 80% formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, and 1 mM EDTA for 3 h at 52 °C (12). After precipitation of the DNA-RNA hybrid, it was collected by centrifugation, and the pellet was dissolved in reverse transcriptase buffer containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 6 mM MgCl2, and 10 mM dithiothreitol. The primer was extended with 20 units of reverse transcriptase, using a mixture was diluted 10-fold in ice-cold S1 buffer (0.25 M NaCl, 0.03 M Na acetate, pH 7.9, and 50% formamide, 0.1 M Tris-HCl, pH 7.9, and 0.5 M NaCl for 10 min at 80 °C. RNA was then added and annealed at 52 °C for 3 h in 60% formamide, 0.5 M NaCl, and 0.1 M Tris-HCl, pH 7.9. Samples were mounted for electron microscopy by the method described by Tilgham et al. (13). Double stranded circular SV40 DNA was included as an internal standard.

Electron Microscopic Analysis—To analyze the exon-intron structure of the genomic clones, R loops were prepared as described below. Heteroduplexes between the DNA of recombinant clones and charon 4A were denatured in 50% formamide, 0.1 M Tris-HCl, pH 7.9, and 0.5 M NaCl for 1 h at 65 °C. The reaction was neutralized, and the DNA was precipitated by alcohol. The DNA was analyzed on a 7 M urea 8% polyacrylamide gel to determine the size of the extended product.

RESULTS

Isolation of Genomic Clones—We have previously isolated and characterized a cDNA clone, pRcO12, which is complementary to the 5' terminal part of the rat pro-α1(II) chain mRNA (7). This cDNA clone was used as a hybridization probe to screen a rat genomic library. Five positive clones were isolated. Three of them contained identical inserts. The three of nonidentical clones, GRC2-1, GRC2-2, and GRC2-3, were further characterized by restriction cleavage mapping and electron microscopic analysis of heteroduplexes. The restriction map for these clones is shown in Fig. 1A. To determine that the clones contain a DNA segment coding for type II collagen, a 4-kb EcoRI fragment from GRC2-1 was labeled with 32P-deoxynucleotide and hybridized with rat chondrosarcoma poly(A) RNA (Fig. 2). The DNA fragment hybridized to a single RNA species of approximately 5.5 kb. The same size RNA hybridized to the type II collagen cDNA (data not shown). Final identification of the clones as coding for type II collagen was obtained by sequence analysis as described later.

Fig. 1. A, restriction endonuclease cleavage map of three overlapping genomic clones. Restriction enzyme cleavage sites are indicated as follows: E = EcoRI; H = HindIII. Overlapping regions within these clones are confirmed by the electron microscopic study (data not shown). B, schematic representation of the rat pro-α1(II) chain gene. Exons are indicated by bars. Exon numbers are given.

Fig. 2. Northern hybridization. RNA from rat chondrosarcoma was separated on a formaldehyde-agarose gel and transferred to nitrocellulose paper. Hybridization was performed as described under "Experimental Procedures." Lane 1, poly(A+) RNA. Lane 2, poly(A-) RNA. The DNA probe was a 4-kb EcoRI fragment of pRgO12.

Three types of experiments were carried out to exclude the possibility that we had isolated fragments of different collagen genes which could contain sequences in common with the hybridization probe we used. Heteroduplex analysis between pairs of overlapping clones clearly showed that the overlapping segments were homologous over the entire length of the overlap and were not interrupted by nonhomologous regions (data not shown). Restriction enzyme analysis also indicated that the overlapping clones contained identical restriction sites at the same location in their homologous segments. Furthermore, R-loop analysis described in the next section showed the common exon-intron structures in the overlapping regions between two clones. Hence, we conclude that these three clones shown in Fig. 1A span a 20-kb segment of contiguous DNA sequence in mouse genome.

The genomic clones were mapped by Southern blotting using segments from the cDNA clone, pRcO12, for the 5' end of the mRNA of the pro-α1(II) chain. The three probes of the clone are shown in Fig. 3B and include: 1) a 600-bp HindIII-BamHI fragment containing the entire cDNA sequence; 2) a 5' probe, a 150-bp HindIII-Atul fragment containing the 5' untranslated region of the rat pro-α1(II) chain mRNA; 3) a 3' probe, a 150-bp Rsa-BamHI fragment encoding the portions of the NH2-terminal propeptide and telopeptide of the pro-α1(II) chain. As shown in Fig. 3, A–C, the 5' probe hybridized to a 4-kb EcoRI fragment of GRC2-1 and a 2.0-kb EcoRI fragment of GRC2-2 but not to GRC2-3. Hence, these EcoRI fragments should include the 5' end of the gene. The 3' probe
Probe (2) refers to panel shown in Fig. 1B. There are at least 16 exons interspersed in a schematic representation of the rat pro-\(\alpha(I)\) chain gene isments of exon and intron size are shown in Table I. A analyzed by electron microscopy (Fig. 4), and the measure-
ment of R-loop structures are consistently observed in the 5' flanking region as shown by the thin arrows in Fig. 4A. One with a 900-bp loop is positioned about 1 kb from the transcription initiation site. The other, with a 500-bp loop, is located about 200 bp from the initiation site.

**Sequence Analysis of the Promoter Region**—We examined the nucleotide sequence of the promoter region of the pro-\(\alpha(II)\) chain gene. A 4-kb EcoRI fragment of GRC2-1, which contained the 5' end of the gene, was subcloned in plasmid PUC 9 (pRgcol2). To localize the first exon more exactly, the plasmid was digested with restriction enzymes and analyzed by Southern blotting using a 150-bp fragment of the cDNA containing the 5' untranslated region of the type II collagen mRNA as a probe. A 750-bp Hind fragment of the 4-kb subcloned segment was found to hybridize with the probe (data not shown). Hence, this fragment should contain the first exon and the 5' flanking sequence of the gene. Fig. 5B shows the restriction map of a 4-kb EcoRI fragment of GRC2-1 and the sequencing strategy used. The nucleotide sequence containing the first exon and its 5' flanking sequence was determined and is shown in Fig. 5A.

The sequence of nucleotides from +5 to +245 is identical to that of the cDNA (7). The first exon contains the 5' untranslated region and the sequence coding for the signal peptide and a portion of the NH\(_2\)-terminal propeptide. The sequence comparison with cDNA also indicates that the last codon of the first exon is split at the second base. A similar division is found in the first exon of the chick pro-\(\alpha(II)\) chain gene (19).

To define precisely the start site for transcription, we performed a S1 nuclease protection experiment and a primer extension experiment. For the S1 protection experiment, a 500-bp Hind-Smal fragment of pRgcol2 was labeled at its Smal 5' end and hybridized with a preparation of rat chondrosarcoma mRNA as shown in Fig. 6B. For the primer extension experiment, a 35-nucleotide HpaII-Smal fragment, labeled at the Smal 5' end, was used as a primer as shown in Fig. 6B. The S1-protected fragment and the cDNA product extended by the primer were analyzed by coelectrophoresis with the product of the base-specific sequencing reactions of the same fragment used for the S1 protection experiment as shown in Fig. 6A.

The microheterogeneity of the transcript initiation site was observed in both the S1 protection and the primer extension experiments. The initiation site for transcription was located within the sequence +1 to +3 as shown in Fig. 6. Preceding the start site for transcription at −29 to −22 is the sequence 5'-ATATAA-3'. This “TATA” box sequence is typically found in the promoter of RNA polymerase II-transcribed genes (20). There is no obvious “CAT” box sequence between −70 and −120 in the type II collagen gene. There are several GC-rich sequence distal to the TATA box. The hexanucleotide 5'-GGGCGG-3' is found at three different places: −73 to 78, −110 to −115, and −187 to −192. The inverted complement sequence of this hexanucleotide, 5'-CCGCCG-3', is located at −217 to −222 and −444 to −449. The presence of the identical
FIG. 4. Electron microscopic analysis of the rat pro-α1(II) chain gene. Single stranded DNA from genomic clone and charon 4A were hybridized with sucrose gradient-enriched mRNA from the rat chondrosarcoma. After staining, 100–1000 fields were examined, photographed, and analyzed. The thick arrow indicates the 5' end of gene. The exon numbers are given. The thin arrows indicate the stem-loop structure in the 5' flanking region, reproducibly observed. A, GRC2-1; B, GRC2-3.

TABLE I
Size of exons and introns in overlapping clones of the type II collagen gene. Results are mean ± S.D.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Length of exon</th>
<th>Length of intron</th>
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<tbody>
<tr>
<td></td>
<td>bp</td>
<td>bp</td>
</tr>
<tr>
<td>1</td>
<td>281 ± 98</td>
<td>6089 ± 242</td>
</tr>
<tr>
<td>2</td>
<td>69 ± 15</td>
<td>101 ± 47</td>
</tr>
<tr>
<td>3</td>
<td>54 ± 19</td>
<td>94 ± 38</td>
</tr>
<tr>
<td>4</td>
<td>89 ± 42</td>
<td>894 ± 296</td>
</tr>
<tr>
<td>5</td>
<td>101 ± 23</td>
<td>704 ± 133</td>
</tr>
<tr>
<td>6</td>
<td>103 ± 21</td>
<td>459 ± 106</td>
</tr>
<tr>
<td>7</td>
<td>60 ± 25</td>
<td>68 ± 27</td>
</tr>
<tr>
<td>8</td>
<td>71 ± 23</td>
<td>777 ± 163</td>
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<tr>
<td>9</td>
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<td>78 ± 30</td>
<td>196 ± 78</td>
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<tr>
<td>12</td>
<td>80 ± 57</td>
<td>2079 ± 126</td>
</tr>
<tr>
<td>13</td>
<td>100 ± 74</td>
<td>316 ± 22</td>
</tr>
<tr>
<td>14</td>
<td>79 ± 38</td>
<td>1100 ± 132</td>
</tr>
<tr>
<td>15</td>
<td>101 ± 39</td>
<td>137 ± 63</td>
</tr>
<tr>
<td>16</td>
<td>104 ± 62</td>
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hexanucleotide and of its inverted complement sequence has been reported in the promoter region of the herpes virus tk gene (21). The sequence 5'-GTGGTTAGA-3' located at −274 to −282 is also identical to the core sequence that has been reported as enhancer element in both viral and cellular genes (22).

DISCUSSION

We have isolated several genomic clones containing the rat type II collagen gene and its 5' flanking region. The gene structure was examined by electron microscopic R-loop analysis. The overlapping clones encompass 15 kb of the 5' terminal part of the type II collagen gene and 5 kb of its 5' flanking region. Because the pro-α1(II) chain mRNA is 5.5 kb long and the total length of the exons (based on the R-loop analysis) is 1.5 kb, these clones encode about one-third of the α1(II) chain. We have also determined the nucleotide sequence surrounding the 5' end of the gene as well as the start site for the transcript.

The gene for the chick pro-α2(I) chain is about 38 kb in size (14), and the gene for the chick pro-α1(III) chain is more than 40 kb long (23). The electron microscopic R-loop analysis reveals that the coding information of the pro-α1(II) chain gene is subdivided into small exons with introns of various sizes. Similar exon-intron structure has been found in the chick pro-α2(I) (14, 15), pro-α1(III) (23), and human pro-α1(I) chain genes (18). A large intron, 6 kb in length, interrupts the first and second exon in the pro-α1(II) chain gene. The existence of an 11-bp exon located proximally to the first exon has been shown in the chick pro-α2(I) chain gene (24). A similar situation could be possible in the pro-α1(II) chain.
gene. Such a small exon would not be detected by electron microscopic analysis and might occur in the 6-kb segment of the type II collagen gene.

Microheterogeneity of the initiation site for transcription is shown in Fig. 5. Because the results of the S1-nuclease protection assay agree very well with the results of the primer extension experiment, it is likely that three different but adjacent sites are used as initiation sites for transcription of the pro-al(I1) chain gene. The assignment of the initiation site is consistent with the sequence of the cDNA clone (7).

The first exon of the pro-al(I1) chain gene contains about 90 bp of untranslated region, and a 85-bp sequence coding for the signal peptide, and a portion of the NH2-terminal propeptide. A similar organization of the first exon was found in the chick pro-al2(I) chain gene. In this case, the untranslated segment is 153 bp long and the coding segment contains 70 bp.

In the region preceding the transcription initiation sites, the two genes diverged.

The most 5' end of the cDNA corresponds to the nucleotide +3 in the genomic DNA. Sequence comparison with the cDNA reveals that the last codon of the first exon is split at the second base. An analogous situation has been reported in the chick pro-al2(I) chain gene (19). Possibly the event that split the first exon may have taken place before the two genes diverged.

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mechanism by which CI repression of bacteriophage λ activates its maintenance promoter. It is of interest to note that the sequence 5'-CCGCC-3' has been found in the 21-bp repeat of the SV40 genome (26, 27) and in the promoter region of the hydroxymethylglutaryl-CoA reductase gene (28). Each of the 21-bp repeats of the SV40 genome contains two copies of the sequence 5'-CCGCC-3' which interact with a cellular transcription factor, Sp1, that is necessary for transcriptional activity (29, 30). The putative promoter region of the hydroxymethylglutaryl-CoA reductase gene is rich in G + C residues (65%) and contains three repeats of the sequence 5'-CCGCC-3'. Both genes are finely regulated by a feedback mechanism. Transcription of the SV40 early gene is repressed by the large T antigen (31). The rate of transcription of the hydroxymethylglutaryl-CoA reductase is suppressed by cholesterol, which is one of the end products in a catabolic pathway involving the reductase (32). Since the biosynthesis of the type II collagen involves many steps of post-translational modification, including processing of pre-procollagen molecules, it is attractive to speculate that expression of the pro-α1(II) chain gene might be regulated by negative feedback at the level of transcription. It is reasonable to assume that the hexanucleotide sequences found in the presumptive promoter of the pro-α1(II) chain gene could play a role in determining the level of the expression of this gene, similar to that proposed for the tk gene.

Two stem-loop structures were observed in the 5' flanking region of the pro-α1(II) chain gene by the electron microscopic heteroduplex analysis. One of them has a loop of 900 bp and is located at approximately 1 kb from the transcription start site. The other, with a loop of 500 bp, is positioned at about 200 bp from the start site. These structures suggested the presence of inverted repeat sequences. The DNA sequence analysis revealed the presence of several inverted repeat sequences in the putative promoter region of the pro-α1(II) chain gene. One of them, 19 nucleotides long (–258 to –276), showed high inverse complementarity to the sequence at position –684 to –703. These sequences could form stable hairpin structures, corresponding to one of the structures observed by electron microscopy.

The sequence 5'-GTGTTAGA-3' is located at –274 to –282 and is identical to the enhancer "core" sequence. This sequence is located close to the putative hairpin structure mentioned above. Enhancers induce high levels of expression of viral and cellular genes, relatively independently of their position and orientation (29). The molecular mechanism of enhancer is still unknown, although several models have been proposed. Viral enhancers generally are located in the regions of DNasel-hypersensitive sites, which correspond to the regulatory region of actively transcribed genes (22). This suggests that both this enhancer sequence and the hairpin structure may alter chromatin structure cooperatively to create regions of increased transcriptional activity. The recent description of a tissue-specific enhancer element in mouse immunoglobulin gene intron suggests that the presence or absence of enhancer-binding proteins determines whether an enhancer functions in a given cell type or not (33–35). They may be important as a binding site for protein factors which determine the tissue-specific expression of the pro-α1(II) chain gene.

There is no significant homology between the promoter sequence of the rat pro-α1(II) chain gene and other collagen genes, including the mouse pro-α2(I) (36), chick pro-α2(I) (37), and the mouse pro-α1(I) chain genes (17). A comparison of the promoter regions of other collagen genes shows that the –50 to –200 region of all four genes is GC-rich (rat α1(II), 72%; mouse α1(I), 68%; chick α2(I), 82%; mouse α2(I), 69%). The divergence of the nucleotide sequence in the promoter region between the pro-α1(II) chain and that of the genes of type I procollagen could be a reflection of factors controlling the tissue-specific expression of these genes.

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Promoter Sequence of the Type II Procollagen Gene