Use of a New Rat Chondrosarcoma Cell line to Delineate a 119-Base Pair Chondrocyte-specific Enhancer Element and to Define Active Promoter Segments in the Mouse Pro-α1(II) Collagen Gene*

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Krish Mukhopadhyay‡§, Véronique Lefebvre‡§, Guang Zhou‡, Silvio Garofalo‡¶, James H. Kimura**+, and Benoit de Crombrugghe‡‡‡

From the ‡Department of Molecular Genetics, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030 and the **Section of Biochemistry, Bone and Joint Center, Henry Ford Hospital, Detroit, Michigan 48202 and the ***Section of Biochemistry, Bone and Joint Center, Henry Ford Hospital, Detroit, Michigan 48202

We show that a new rat chondrosarcoma (RCS) cell line established in long-term culture from the Swarm tumor displayed a stable differentiated chondrocyte-like phenotype. Indeed, these cells produced the collagen types II, IX, and XI and alcian blue-stainable cartilage-specific proteoglycans, but no type I or type III collagen. To functionally characterize their chondrocytic nature, the cells were stably transfected with a type II collagen/geo chimeric gene which confers essentially perfect chondrocyte-specific expression in transgenic mice. RCS cells expressed both β-galactosidase and G418 resistance, in comparison with similarly transfected 10T1/2 and NIH/3T3 fibroblasts which did not. These cells were then used to perform a systematic deletion analysis of the first intron of the mouse type II collagen gene (Col2a1) using transient expression experiments to determine which segments stimulated expression of a luciferase reporter gene in RCS cells but not in 10T1/2 fibroblasts. Cloning of two tandem copies of a 156-base pair (bp) intron 1 fragment (+2188 to +2343) in a construction containing a 314-bp Col2a1 promoter caused an almost 200-fold increase in promoter activity in RCS cells but no increase in 10T1/2 cells. DNase I footprint analysis over this 156-bp fragment revealed two adjacent protected regions, FP1 and FP2, located in the 3′-half of this segment, but no differences were seen with nuclear extracts of RCS cells and 10T1/2 fibroblasts. Deletion of FP2 to leave a 119-bp segment decreased enhancer activity by severalfold, but RCS cell specificity was maintained. Further deletions indicated that sequences both in the 5′ part of this 119-bp fragment and in FP1 were needed simultaneously for RCS cell-specific enhancer activity. A series of deletions in the promoter region of the mouse Col2a1 gene progressively reduced activity when these promoters were tested by themselves in transient expression experiments. However, these promoter deletions were all activated to a similar level in RCS cells by a 231-bp intron 1 fragment that included the 156-bp enhancer. The RCS cell-specific activity persisted even if the Col2a1 promoter was replaced by a minimal adenovirus major late promoter. This 231-bp intron 1 fragment also had strong enhancing activity in transiently transfected mouse primary chondrocytes. Our experiments establish the usefulness of RCS cells as an experimental system for studies of the control of chondrocyte-specific genes, provide an extensive delineation of segments in the Col2a1 first intron involved in chondrocyte-specific activity, and show that promoter sequences are dispensable for chondrocyte specificity.

The differentiation of mesenchymal cells into chondrocytes results in the synthesis and secretion of a series of proteins characteristic of the extracellular matrix of cartilages. These include types II, IX, and XI collagens, the proteoglycan aggregan, link protein, and cartilage matrix protein, the expression of which is part of a genetic program of differentiation specific for chondrocytes (1, 2). Earlier studies on the biosynthesis of these cartilage components have used mainly primary chondrocytes, but the phenotypic instability of these cells, which varies with culture conditions, has made experiments on chondrocyte differentiation difficult (3–6). A small number of chondrocyte cell lines that maintain at least part of the chondrocyte phenotype during culture have been isolated recently (7–12). Such stable chondrocyte cell lines are essential for achieving further understanding of the genetic program of chondrocyte differentiation.

Because the gene for type II collagen is expressed at very high levels in chondrocytes (2, 13, 14), it is an excellent candidate for study of chondrocyte differentiation. Humans and mice with mutations in type II collagen often show severe cartilage defects and skeletal malformations (15–17). During embryonic development, the gene becomes active in cartilage anlagen as early as the time of mesenchymal condensation preceding cartilage formation. Hence, type II collagen can be considered an early and abundantly expressed marker of chondrocyte differentiation. Although the gene for type II collagen is also expressed transiently in some extrachondrogenic sites during embryonic development (14), this occurs at much lower levels, and the significance of this extrachondrocytic expression is not understood, in contrast to the richly documented role played by this molecule in cartilage function.

Our long-term goal is to identify and characterize the DNA-binding proteins involved in the chondrocyte-specific activity of the type II collagen gene. In this study we sought to better delineate sequences within the mouse type II collagen gene

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§ These two authors have equally contributed to this manuscript.

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‡ Present address: Research Dept., Shriners Hospital for Crippled Children, 3101 S. W. Sam Jackson Park Rd., Portland, OR 97201.

** To whom correspondence should be addressed: Dept. of Molecular Genetics, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-792-2590; Fax: 713-794-4298.
Delineation of a 119-bp Col2A1 Enhancer

(Col2a1)\(^1\) that can confer chondrocyte-specific expression to reporter genes. Previous transient expression experiments using primary chick chondrocytes had identified a 620-bp chondrocyte-specific enhancer in the first intron of the rat Col2a1 gene (3). Subsequently, similar experiments showed that a 380-bp segment within this 620-bp fragment activated expression of a reporter gene 11-fold in chondrocytes and that a 260-bp subfragment activated expression 6-fold (4). A point mutation in this 260-bp fragment decreased this activity by about two-thirds. In other experiments, two silencer elements identified in the rat Col2a1 promoter were proposed to participate in inactivating the gene in nonchondrocytic cells (18).

In the present study we first examined the expression of a series of molecular markers to characterize the chondrocytic phenotype of a new rat chondrosarcoma (RCS) cell line derived from the Swarm chondrosarcoma tumor after long-term culture. The cells were further characterized by comparing them with 10T1/2 and NIH/3T3 fibroblasts after stable transfection with a mouse Col2a1 chimera DNA construction and by establishing that the Col2a1-derived transgene was selectively expressed in chondrocydona cell line which were used in transient expression experiments to delineate minimal sequences in the first intron of the mouse Col2a1 gene that were needed for chondrocyte-specific enhancer activity and to determine active sequences in the promoter of this gene.

MATERIALS AND METHODS

Oblention of the Rat Chondrosarcoma Cell Line—RCS cells were obtained from the National Cancer Institute (NCI) and derived from a rat chondrosarcoma (19) that were obtained after long-term suspension culture. These nodules which initially contained primarily chondrocyte-like cells but also few fibroblast-like cells were dispersed in trypsin and collagenase. The fibroblastic components were removed by repetitive monolayer culture from which a population of less adherent chondrocyte-like cells was isolated. These cells were continuously propagated in serial monolayer culture for several years and constitute the RCS cell line used in the present study. The cells show a doubling time of less than 24 h and display a completely stable phenotype under standard culture conditions.

Other Cells—10T1/2, NIH/3T3, and C57/129 cell lines were purchased from the American Type Culture Collection. Primary chondrocytes were prepared at 1 to 2 weeks old from the cartilages of 3-week-old rats as described previously (20). Rat osteosarcoma cells (ROS 17/2.8) were kindly provided by Dr. W. T. Butler (Department of Biological Chemistry, University of Texas Health Science Center, Houston, TX).

Cell Cultures—All cell types were cultured at 37 °C under 5% CO\(_2\) in Dulbecco's modified Eagle's medium (high glucose, without pyruvate, Life Technologies, Inc.) supplemented with penicillin (50 units/ml), L-glutamine (2 mM), and 10% heat-inactivated fetal calf serum (Life Technologies, Inc.). Cell passaging was performed using trypsin-EDTA.

Northern Blot Analysis—Isolation of total RNAs, Northern blot analysis, and preparation of labeled DNA probes were performed as described previously (12, 20).

Staining with Alcian Blue—Staining of cartilage-specific proteoglycans in cell culture monolayers was done with alcian blue at pH 1 as described previously (12, 20).

Analysis of Collagens—Labeling of cartilages synthesized by cells in culture was performed at an efficiency for 40 h in fresh culture medium supplemented with 15 μCi/ml of L-[2,3,4,5-\(^3\)H]proline (Amersham), 50 μg/ml ascorbic acid, 100 μg/ml 4-aminoquinoline urea, 1 mM cysteine, and 1 mM pyruvate. Culture media were collected, centrifuged, and acidified with 0.5 M acetic acid. Monolayers containing the cells and their extracellular matrix were harvested in 0.5 M acetic acid. Pepsin at 0.25 mg/ml was added to these culture samples, and incubation was carried out for 4 h at 15 °C. Pepsin was inhibited by raising the pH to 8 with NaOH and 50 mM Tris, and by addition of 10 μg/ml pepstatin. Aliquots of the samples were digested with 25 μg/ml purified bacterial collagenase (type VII; Sigma) in the presence of 2 μg CaCl\(_2\), 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin for 2 h at 37 °C. Collagens in all samples were precipitated with 30% ammonium sulfate, and with 80% ethanol, and subjected to polyacrylamide gel (6%) electrophoresis after boiling for 2 min in loading buffer supplemented or not with 100 mM dithiothreitol. Prestained protein molecular weight standards were from Life Technologies, Inc.

Gels were treated with Amplify (Amersham), and x-ray films were exposed for 1–3 days.

Transient Transfections with a Col2a1/geo Chimeric Transgene—Two different plasmids were used for transient transfections. The plasmid pPGK-HYG contains a hygromycin (HGP) resistance gene driven by a phosphoglycerate kinase-1 (PGK) promoter (22). The plasmid pCol2a1/geo was constructed in pBlueScript II KS (+/−) (Stratagene) by cloning a 7.2-kb fragment of the mouse Col2a1 gene upstream of a SA-jugeo-bpA cassette.\(^2\) The Col2a1 fragment contained 3 kb of promoter sequences, exon 1, and 3034 bp of intron 1. It was released from a cosmid vector (23) in two pieces by digestion with NotI/Sal and SalI/XbaI and reconstituted in Bluescript by cloning at the NotI and XbaI sites. The Col2a1 translation initiation codon (ATG) was changed to CTG by PCR mutagenesis. The SA-jugeo-bpA cassette was isolated from the plasmid directed both β-galactosidase expression and hygromycin B resistance in transient transfection experiments. A splice acceptor (SA) was present at its 5′ end and the bovine growth hormone gene polyadenylation signal (bpA) at its 3′ end. The cassette was released from the pSA-jugeo plasmid (24) by digestion with SspI and Asp-718 and cloned at corresponding sites in pBlueScript.

Permanent transfections were performed by the modified DNA-calcium phosphate coprecipitation method described by Chen and Okayama (25). 5 × 10\(^5\) cells were plated in 50-cm\(^2\) tissue culture dishes and transfected with 1 μg of pPGK-HYG alone or together with 20 μg of pCol2a1/geo. Hygromycin-resistant cells were selected for 10 days with 200 μg/ml hygromycin B. Southern analysis was performed on the pools of hygromycin-resistant cells to verify integration of the Col2a1 transgene. This was done by using the 3-kb Escherichia coli β-galactosidase gene (lacZ) as a probe. Selection of cells expressing the neomycin resistance gene was performed by treatment with G418 at 300 or 500 μg/ml as indicated. β-Galactosidase activities were measured with a chemiluminescent assay kit (Tropix, Bedford, MA), and protein was assayed with the Bradford reagent (Bio-Rad).

DNA Constructions Used in Transient Transfections—Col2a1-promoter constructs were constructed in the pA3LUC vector (26). For some constructions, a modified version of this plasmid (pLuc4) was used in which a multiple cloning site was introduced immediately upstream of the luciferase gene (27). Pieces of the Col2a1 gene were amplified by a combination of PCR and intron fragments were generated by PCR using two oligonucleotide primers containing a Hind II recognition site at its 5′ end, T1 to T17 intron 1 fragments were generated by PCR using two oligonucleotide primers containing a Hind II recognition site at its 5′ end, and the other a Bgl II site at its 5′ end. Some of these intron fragments were cloned as two or four tandem repeats as indicated in the figure legends. In order to form these repeats, the PCR products were digested with Bgl II and Bgl II, ligated, and treated again with BamHI and Bgl II to keep only multimers ligated head to tail. Dimer or tetramers were isolated by electrophoresis in agarose gel and ligated to the appropriate vectors as indicated in the figures. Intron fragments were cloned in their natural 5′ to 3′ orientation. To construct the padML, a 65-bp fragment of the adenovirus type 2 major late promoter (−46 to −100) was isolated by EcoRI and SmaI digestion from the pUC19 plasmid, a Hind II linker was added to the SmaI site, and the EcoRI site was blunt-ended. This promoter fragment was cloned in the pA3LUC vector between the blunt-ended KpnI and Hind II sites. The sequences of all constructs were verified by dye dideoxy DNA sequencing.

Transient Transfections—DNA transfection experiments were performed with RCS, 10T1/2, and C57 cells carried out by electroporation of cells harvested near midlog growth phase. RCS cells were detached from plastic and freed of their abundant extracellular matrix by treatment for 1 h at 37 °C with 1.5 mg/ml bacterial collagenase (collagenase D, Boehringer Mannheim) in Dulbecco's modified Eagle's medium, followed by two washes in phosphate-buffered saline. 10T1/2 and C57 cells were used for various experiments.
cells were harvested with trypsin/EDTA. 2 × 10⁶ cells were distributed in 0.4-mI electroporation cuvettes in 200 μl of phosphate-buffered saline with 10 μg of pBluescript II KS (28) was used as an internal control of transfection efficiency. RCS cells were electroporated at 250 V, 500 μF at 4 °C, and 10T1/2 and C5C12 cells at 300 V, 500 μF at room temperature, using a Gene Pulser apparatus (Bio-Rad). Cells were replated in 20-cm² plastic dishes, medium was changed within 24 h, and the cultures continued for another 24–48 h. Primary chondrocytes were transfected by DNA:RNA coprecipitation (25) within 24 h after release from cartilage. Five million cells were plated per 50-cm² dishes and transfected using 18 μg of Col2a1-luciferase plasmids, and 6 μg of pSV2βgal plasmid. Twenty-four h before harvesting the primary chondrocytes, 50 μg/ml ascorbic acid was added to the culture media in order to stimulate the expression of the differentiated phenotype (20), together with 1 mg cysteine and 1 mg pyruvate. Luciferase assays were performed according to Wood et al. (26) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) and β-galactosidase assay as substrate.

RESULTS

Phenotype of the Rat Chondrosarcoma Cell Line—Fig. 1 shows that RCS cells exhibited a polygonal or round shape in both nonconfluent (A) and confluent (B) cultures. They secreted a very abundant extracellular material which stained intensely with alcian blue (Fig. 1C), indicating the presence of large amounts of cartilage-specific proteoglycans. Fig. 2A shows that the cells contained large amounts of type II procollagen RNA (lane 2), although the hybridization signal was clearly less intense than in primary mouse rib chondrocytes (lane 1) and primary rat articular chondrocytes (not shown). The cells also contained RNA for α2(IX) procollagen, another characteristic chondrocyte-specific marker, showing a signal that was stronger in intensity than that found for primary mouse chondrocytes (Fig. 2B). No RNA for type X collagen, a characteristic marker of hypertrophic chondrocytes, could be detected in the RCS cells, whereas it was found in mouse rib primary chondrocytes, which presumably contained a substantial proportion of hypertrophic cells (Fig. 2C). Only a weak signal for type X collagen transcript was detected in the rat chondrocytes, most probably due to their articular cartilage origin. Importantly, no RNA for α1(I) procollagen was observed in RCS cells unlike what is seen in primary chondrocytes after a short time in culture (Fig. 2C). In contrast, the mouse pro-α1(I) probe hybridized strongly with RNA isolated from rat primary chondrocytes and rat osteosarcoma cells. No RNA for either osteocalcin or osteopontin was detected, but a probe for matrix Gla protein gave a strong signal (Fig. 2D). Fig. 3 shows that RCS cells, like mouse primary chondrocytes, synthesized large amounts of type II collagen whose α1 chain characteristically migrated slightly slower than the α1(I) collagen chain, as well as type XI collagen whose α1 and α2 chains migrated more slowly than the α1(I) collagen chain, whereas its α3 chain comigrated with the α1(I) collagen...
proline and further processed for the analysis of collagens as described under "Materials and Methods." Aliquots from the culture media and from the cell and matrix layers of each culture were treated with pepsin only (C and M, respectively) or with pepsin followed by bacterial collagenase (C* and M*, respectively). SDS-PAGE were run without (A) or with dithiothreitol (B). Numbers indicate the molecular mass of protein standards which were run alongside on the same gels.

Collagen chains produced by 10T1/2 cells were separated into different bands as follows: a, α1(III) trimer; b, α1(V); c, α1(I) and probably α2(V); d, α2(I); e, α2(V); f, α1(XI); g, α2(XI); h, α1(II) and probably α3(XI); i, l, and m, likely type IX collagen chains undigested or partially digested by pepsin.

FIG. 3. Analysis of collagens produced by RCS cells. Confluent cultures of 10T1/2 fibroblasts, RCS cells, and mouse primary chondrocytes (MPC, 20 h after release from cartilage) were labeled with [3H]proline and further processed for the analysis of collagens as described under "Materials and Methods." Aliquots from the culture media and from the cell and matrix layers of each culture were treated with pepsin only (C and M, respectively) or with pepsin followed by bacterial collagenase (C* and M*, respectively). SDS-PAGE were run without (A) or with dithiothreitol (B). Numbers indicate the molecular mass of protein standards which were run alongside on the same gels.

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Both collagen types accumulated more abundantly in the cell and matrix layers than in the culture media. RCS cells also produced a ladder of pepsin-resistant polypeptides with an apparent molecular mass comprised between 150 and 250 kDa under nonreducing conditions. These polypeptides which were sensitive to bacterial collagenase most likely correspond to type IX collagen chain trimers undigested or partially digested by pepsin. After reduction, they gave rise to smaller collagenase-sensitive peptides with apparent molecular mass of 86, 70, and 60 kDa which likely correspond to intact or partially digested type IX collagen chains (32). Trimmers of α1(III) chains and α2(I) chains, which were the major pepsin-resistant polypeptides in 10T1/2 cell cultures, were undetectable in the RCS cell samples.

Altogether, these results indicated that the RCS cells express a number of characteristic chondrocyte differentiation markers, including type II, IX, and XI collagens, and cartilage-specific proteoglycans. The absence of type X collagen RNA suggests that they were frozen in a state before transition into hypertrophic chondrocytes. Importantly, the complete absence of type I collagen indicates that these cells, unlike other chondrocytic cell lines previously described (7-11), do not express a partial chondrocyte or fibroblastic phenotype.

Stable Transfection of RCS, 10T1/2, and NIH/3T3 Cells with a Chimeric Col2a1 Construct—To further characterize RCS cells, we compared these cells with 10T1/2 and NIH/3T3 fibroblasts after stable transfection with a Col2a1-geo chimeric construct containing 3 kb of 5' flanking sequences of the mouse pro-α1(II) collagen gene, exon 1, and about 3 kb of intron 1, linked to the bgeo reporter gene (Fig. 4A). The bgeo gene encodes a fusion polypeptide that includes both E. coli β-galactosidase and neomycin resistance activities. In other experiments, we showed that this construction directed essentially perfect chondrocyte-specific β-galactosidase activity in transgenic mice. RCS cells, 10T1/2, and NIH/3T3 fibroblasts were stably transfected with this construction together with an expression vector containing a hygromycin resistance gene driven by a phosphoglycerate kinase promoter (pPGK-HYG).

After a first selection of the cells with hygromycin, a Southern hybridization with the DNA of the pooled colonies showed a signal of about equal intensity for the three cell types, indicating that the Col2a1 transgene had been stably integrated (data not shown). To determine whether the Col2a1 transgene...
was expressed, the pooled colonies were first tested for resistance to neomycin. As shown in Fig. 4B, RCS cells continued to grow in the presence of G418, whereas 10T1/2 and NIH/3T3 fibroblasts rapidly died. In control experiments, RCS cells that did not contain the Col2a1-βgeo transgene died within a few days in G418 selection medium (data not shown). Results similar to those in Fig. 4B were also obtained when a lower G418 concentration (350 instead of 500 μg/ml) was used (data not shown).

We then tested expression of β-galactosidase in RCS, 10T1/2, and NIH/3T3 cells stably transfected with pPGK-HYG alone or with both pPGK-HYG and Col2a1-βgeo (Fig. 4C). After selection with hygromycin, no difference in β-galactosidase activity was observed whether 10T1/2 and NIH/3T3 fibroblasts were transfected with one or with both plasmids. RCS cells contained endogenous β-galactosidase activity that was considerably higher than that of 10T1/2 and NIH/3T3 cells. However, the RCS cell β-galactosidase activity was much higher in cells containing the Col2a1-βgeo construct than in control cells. After G418 selection, these levels increased by an order of magnitude in RCS cells transfected with the Col2a1-βgeo construct. This increase was probably due to the G418 selection which eliminated cells that were transfected by the hygromycin vector only and probably also the cells that expressed levels of βgeo below the G418 threshold. These functional experiments indicated that RCS cells were able to support the activity of a stably transfected chondrocyte-specific chimeric transgene whereas in 10T1/2 and NIH/3T3 cells the transgene was inactive. These results are consistent with the notion that, unlike 10T1/2 and NIH/3T3 fibroblasts, RCS cells contain the transcription factors needed for the activity of a chondrocyte-specific transgene.

Initial Identification of Sequences in Intron 1 of the Col2a1 Gene That Show Enhancer Activity Specifically in Chondrosarcoma Cells—RCS cells were then used for transient expression experiments with the DNA constructions shown schematically in Fig. 5. An 886-bp fragment of the mouse Col2a1 gene containing 767 bp of 5′-flanking promoter sequences and 119 bp of exon 1 was placed upstream of the luciferase reporter gene in the pA2LUC vector. Various deletion fragments of intron 1 were cloned in the pPGK-HYG vector. All these DNA constructs were transiently transfected into 10T1/2, C2C12, and RCS cells. Luciferase activities were measured in cell extracts and normalized for transfection efficiency (see "Materials and Methods"). Transcriptional activation of the promoter induced by intron fragments is given relative to the activity obtained with the pP1L construct (considered as 1) and is presented as the average ± S.E. of all transfection assays performed, with the number of assays indicated in parentheses.
cells but that a 908-bp fragment (+2344 to +3251) located 3' of this segment was much less active. Further work was focused on smaller fragments. A 546-bp fragment (+1880 to +2425) showed a 14-fold increase specifically in chondrosarcoma cells but smaller fragments were clearly less active. However, we found that when two copies of a 465-bp fragment (+1880 to +2343) were cloned tandemly a 44-fold increase in activity was produced in the other two cell types. Similarly, two tandem copies of a fragment containing FP2 were inactive (Fig. 3).

Fig. 6. Protein binding and nucleotide sequence analysis of the 231-bp Col2a1 enhancer. In A, a DNase I protection assay was performed on the coding strand of the Col2a1 231-bp enhancer fragment in the absence of nuclear extracts (no N.E.) or after preincubation with nuclear extracts from 10T1/2 or RCS cells, as indicated at the top of the lanes. The first lane shows a G ' A sequencing of the same DNA strand. Two regions protected by nuclear proteins are bracketed and labeled FP1 and FP2. In B, the coding strand of the 231-bp enhancer is aligned with analogous regions in the human and rat type II collagen genes. Only 96 bp of the corresponding rat sequence are available from the GenBank™ database. Only bases in the human and rat sequences that differ from the mouse sequence are shown; identical bases are indicated by a dot. Gaps, marked by a dash, have been added to maximize alignment. A decamer sequence in the rat gene, that was postulated to have a role in chondrocyte-specific expression, is shown in italics. The regions footprinted in DNase I protection assays are underlined in the mouse sequence and labeled FP1 and FP2. The numbers +2113 to +2344 above the mouse sequence indicate the distance in nucleotides from the mouse Col2a1 transcription start site. Repeated DNA sequencing has indicated that nucleotide 2280 in the mouse sequence is a C residue rather than a T residue as reported earlier (23).

DNase I Footprint Studies Using the 231-bp Intron Fragment—The active 231-bp enhancer fragment was used in DNase I footprint experiments to compare the patterns of protection by nuclear proteins of RCS and 10T1/2 cells. Fig. 6A shows two adjacent areas of protection on the upper DNA strand, indicated as FP1 and FP2, which were located toward the 3' end of the 231-bp fragment. No other clear footprints were detected elsewhere in this fragment. Footprints covering the same area of DNA were observed on the lower strand, and again no other footprints were seen elsewhere in this fragment (data not shown). The 3' part of FP1 includes part of a 10-bp sequence (CACAATGCGAT, see Fig. 6B) which in the rat Col2a1 gene was previously implicated in chondrocyte-specific enhancer activity and was reported to be a binding site for a chondrocyte-enriched protein (4). However, as shown in Fig. 6A, we found no difference in FP1 between 10T1/2 and RCS cell nuclear extracts even when different concentrations of nuclear extracts were used. Similarly, FP2 showed no differences with these two extracts. Since the 5' part of FP1 and the protected DNA of FP2 were very GC-rich, it is possible that the footprints were due to GC-binding proteins in extracts of both cell types. Interestingly, most of the region corresponding to FP2 is not well conserved in the human and rat genes (Fig. 6B).

Additional Deletions of the 231-bp Enhancer Fragment—We then asked whether a 97-bp subfragment (+2248 to +2344) of the 231-bp fragment, containing the two protected regions identified by DNase I footprinting, had enhancer activity. When cloned as 2 tandem copies in the 886-bp promoter vector, this 97-bp fragment failed to activate the promoter in transient expression experiments using RCS cells (Fig. 7A). Similarly, four tandem copies of a fragment containing FP1 or four tandem copies of a fragment containing FP2 were inactive (Fig. 7A). Thus, each of the two segments containing the individual footprints as well as a 97-bp fragment containing the two footprints were unable by themselves to act as chondrocyte-specific enhancers.

In subsequent experiments, a shorter 433-bp (-314 to +119) promoter fragment was used to replace the 886-bp (-767 to +119) promoter. The activity of this promoter was reduced about 5 to 10 times compared to the 886-bp promoter in both...
10T1/2 and RCS cells (data not shown), despite the fact that this shorter promoter removed two potential silencer elements that were identified in the rat gene (18). Sequence comparison of the rat, human, and mouse genes failed to show conservation of these silencer elements (33). When two tandem copies of the 231-bp fragment (+2113 to +2343) were cloned upstream of the promoter, an 88-fold activation was seen in transient expression experiments using RCS cells and no activation using 10T1/2 cells (Fig. 7B). Additional deletions of the 231-bp enhancer fragment were tested in constructions containing two tandem copies of these subfragments. A 5' deletion producing a 156-bp fragment resulted in even greater activity than the 231-bp fragment in RCS cells (Fig. 7B). When 37 bp at the 3' end of this 156-bp fragment were deleted, which removed most of FP2, the remaining 119-bp fragment was still active in RCS cells albeit at a severalfold lower level. A further 30-bp deletion of 3' sequences in this 119-bp fragment, which removed FP2 and the 3' part of FP1, including the previously described decamer sequence, leaving an 89-bp fragment (+2188 to +2276), resulted in complete loss of activity in RCS cells. Similar results were obtained with a construction that deleted both 5' and 3' sequences (+2232 to +2285).

Hence, the RCS-specific enhancer was located in a 156-bp fragment in which both 5' and 3' sequences appeared to be required simultaneously for activity since neither the 3' 97-bp segment, containing FP1 and FP2, nor the 5' 89-bp segment was able to show enhancer activity by themselves. Deletion of the 3'-DNA corresponding to most of FP2 in the 156-bp fragment significantly decreased the activity of the enhancer, but RCS specificity was maintained indicating that the elements necessary for chondrocyte enhancer activity were contained in a 119-bp segment.

Deletions in the Mouse Col2a1 Promoter—To determine whether sequences in the promoter were needed for RCS cell-specific expression and also to identify sequences in the promoter that were important for its activity, a series of promoter deletions was generated (Fig. 8). Like the –767 to +119 promoter (Fig. 5), the –314 to +119 and the –314 to +7 promoters by themselves were considerably more active in 10T1/2 cells than in chondrosarcoma cells (Fig. 8). Like a deletion from –767 to –314, which reduced promoter activity severalfold (data not shown), deletions to –159 and to –89 further reduced promoter activities in both cell types, suggesting that the deleted segments contain binding sites for transcriptional activators. Cloning of two tandem copies of the 231-bp intron fragment produced a large increase in activity with each of the different promoter deletions in chondrosarcoma cells (Fig. 8).

The absolute activities of these different promoter-enhancer constructions were similar, resulting in a several thousandfold stimulation with the –159 and –89 promoters. Hence, the promoter sequences upstream of –89 are not needed to achieve optimal activation by the 231-bp enhancer segment in chondrosarcoma cells. In contrast, the 231-bp enhancer had no effect on promoter activity in 10T1/2 cells with the –314 promoter and only a small effect with the two smaller promoters (Fig. 8).

To determine whether any sequences in the –89 Col2a1 promoter were needed for chondrosarcoma cell specificity, the Col2a1 promoter was replaced by a short segment of the adenovirus major late promoter (–46 to +10) (box with grid) inserted into the KpnI and HindIII sites of the pαLuc vector. Plasmids pF9 and pF9L were obtained by cloning, respectively, a 165-bp and a 95-bp promoter fragment (boxes with grid) of the Col2a1 gene into the HindIII site of the pαLuc vector. The sizes of the promoter fragments are indicated inside their respective boxes, and the position of their first and last nucleotides relative to the Col2a1 transcription start site is indicated at the bottom left and right sides of the boxes. Plasmids pF9P3L and pF9P4L, and pF9P5L contain 2 (2×) tandem copies of the 231-bp Col2a1 enhancer (boxes with stripes) inserted in the Smal site of the corresponding promoter-luciferase vectors. These DNA constructs were transiently transfected in RCS and 10T1/2 cells. Luciferase activities are presented as the average ± standard deviation of three independent transfection assays.

**DISCUSSION**

Rat Chondrosarcoma Cells Display a Stable Chondrocytic Phenotype—The new RCS cell line we have used in our experi-
iments has unique properties not previously described in other chondrocytic cell lines. The advantage of this cell line which has been established in long-term culture from cells obtained from the rat Swarm chondrosarcoma tumor is that its chondrocytic phenotype is completely stable in standard tissue culture conditions in contrast to that of primary chondrocytes. Importantly, as for chondrocytes in intact animals, these RCS cells did not contain any type I collagen but expressed the chondrocyte-specific type II, IX, and XI collagens. In addition, they also synthesized large amounts of alcian blue-stainable cartilage-specific proteoglycans. The absence of a 1(X) collagen RNA suggested that these cells were frozen in the pathway of chondrocyte differentiation at a stage preceding transition to hypertrophic cells. These cells were then stably transfected with a chimeric gene in which sequences of the mouse Col2a1 gene drove expression of the βgeo reporter gene, a construction that displayed essentially perfect chondrocyte-specific expression in transgenic mice. The stably transfected RCS cells exhibited clear G418 resistance in contrast to similarly transfected 10T1/2 and NIH/3T3 fibroblastic cells which were unable to survive after G418 addition. This experiment strongly suggested that, like chondrocytes in intact embryos, the chondrosarcoma cells contained transcription factors needed to selectively activate the Col2a1-βgeo chimeric gene, whereas 10T1/2 and NIH/3T3 fibroblasts did not.

**Delineation of Chondrosarcoma-specific Enhancer Sequences in the Mouse Col2a1 Gene**—The RCS cells were then used in transient expression experiments to systematically dissect which of the segments of the mouse Col2a1 gene first intron were able to stimulate the expression of a luciferase reporter gene in chondrosarcoma cells but not in 10T1/2 fibroblasts and C3H10T1/2 myoblasts. We also sought to analyze the effects of successive deletions in the promoter of this gene and to determine whether any Col2a1 promoter sequences were needed for the effects of the intron sequence to appear.

Our experiments demonstrated that the presence of two tandem copies of a 156-bp segment of intron 1 in a construction containing a 314-bp Col2a1 promoter segment provided an almost 200-fold promoter activation in RCS cells but had no effect on promoter activity in 10T1/2 cells. By comparison, previous transient expression experiments from another laboratory using primary chondrocytes indicated that a 260-bp minimal active enhancer sequence of the intron 1 of the rat Col2a1 gene provided a much more modest 6-fold cell-specific enhancement in activity (4). This rat DNA segment contained sequences that are in part homologous to the mouse 156-bp sequence. In transient expression experiments in mouse primary chondrocytes, a construction containing a 231-bp intron fragment, which included the 156-bp fragment, showed a high level of expression of the reporter gene which paralleled its activity in RCS cells. In separate experiments separate from our laboratory, a fragment of 182 bp, which included the 156-bp fragment, conferred essentially perfect chondrocyte-specific expression of a lacZ reporter gene in transgenic mice.2

In the 3′ part of the 156-bp segment, we identified two adjacent DNase I footprints, FP1 and FP2, but these same footprints were found with nuclear extracts from chondrosarcoma cells and 10T1/2 fibroblastic cells. The 5′ footprint (FP1) included part of a decamer sequence that had previously been postulated to be involved in chondrocyte-specific expression of the rat Col2a1 gene (4). In nuclear extracts of primary chondrocytes, Wang et al. (4) identified a protein binding to this decamer sequence that was enriched in chondrocytes, although it did not appear to be completely chondrocyte-specific. Removal of most of the FP2 sequence decreased the activity of the enhancer several fold, but the remaining 119-bp DNA was still RCS cell-specific. Since the FP2 sequence in the mouse gene is not present at an analogous location in the rat and human sequences, we speculate that in these species a similar sequence present at some other location can substitute for this GC-rich sequence. Removal of the sequence of FP1, leaving an 89-bp fragment at the 5′ end of the 156-bp segment, abolished the activity of the enhancer. Importantly, a 97-bp fragment containing the 3′ part of the 156-bp segment including both FP1 and FP2 was also completely inactive. Hence, our deletion analysis indicates that sequences both in the 5′ and in the 3′ portions of this 119-bp fragment are needed together for RCS cell-specific enhancer activity. The 5′-DNA segment of the 119-bp fragment contains an inverted repeat sequence which is completely conserved in the mouse and human genes. The sequence of this inverted repeat contains 11 bp in one arm of the repeat and 10 bp out of 11 that are conserved in the other arm. We propose a model whereby proteins that bind to the 3′ part of the 119-bp segment and proteins that bind to the 5′ part of this segment cooperate with each other either in DNA binding or in transcriptional activation to provide RCS cell-specific enhancing activity.

**Analysis of the Col2a1 Promoter**—Our deletion and substitution experiments using the promoter produced three novel results. First, successive deletions between −314 and −89, which removed two conserved GC-rich segments, indicated that the promoter contained elements between these two endpoints that could activate transcription when the promoter itself was tested in transient expression experiments. We also noted that constructions containing these promoters without intron 1 sequences were much more active in 10T1/2 fibroblasts than in RCS cells, suggesting that the transcription factors that interact with these segments are more active or more abundant in fibroblastic cells than in RCS cells. In transgenic mice, constructions containing either a 767-bp or a 314-bp promoter without intron sequences were unable to direct activity of a reporter gene in chondrocytes. Second, when an active intron enhancer segment was cloned upstream of the promoter, the levels of activity of the promoter were greatly increased in RCS cells, but they were approximately the same with different lengths of promoter. This suggested the hypothesis that the protein interactions needed for optimal activation of the promoter in transient expression experiments occur between enhancer-bound proteins and proteins bound to the promoter segment between −89 and +6. The segment upstream of −89 might be needed to control activation of the endogenous Col2a1 gene in chromatin, to mediate responses to extracellular stimuli, to effect interactions with other parts of the gene, or to regulate expression in nonchondrocytic cells. Lastly, our transient expression experiments demonstrated that no specific elements in the Col2a1 promoter were required for activity of the RCS-specific enhancer. Indeed, the response of a minimal adenovirus major late promoter to the 231-bp enhancer was at least as high as that of Col2a1 promoters, and this construction showed very little activation in 10T1/2 fibroblasts. This conclusion was supported by results obtained in transgenic animals,

**Table 1**

| Constructs | Luciferase activities |
|------------|----------------------|
| pPSL       | 0.34–0.19            |
| pl9P5SL    | 196–222              |

**Delineation of a 119-bp Col2A1 Enhancer**

Mouse primary chondrocytes were transiently transfected with the pPSL and pl9P5L plasmids described in Fig. 8. Luciferase activities are given for one representative experiment done with duplicate cultures.

2Downloaded from http://www.jbc.org/ by guest on April 27, 2019
since mice harboring a construction in which a 182-bp enhancer fragment was cloned upstream of a minimal β-globin promoter displayed high levels of chondrocyte expression of a β-globin reporter gene. 2

In summary, our results establish the unique usefulness of a new RCS cell line as a tissue culture system to study chondrocyte-specific regulatory elements of the Col2a1 gene and presumably other chondrocyte-specific genes. Our experiments indicate that both 5’ and 3’ sequences in a 119-bp intron 1 segment are needed for RCS cell-specific enhancer activity. We also demonstrate that the Col2a1 promoter sequences are dispensable for this cell-specific enhancer activity. Further work is needed to better characterize the binding sites within the 119-bp segment for proteins involved in the chondrocyte-specific expression of the Col2a1 gene and to isolate cDNA clones for these proteins.

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Use of a New Rat Chondrosarcoma Cell line to Delineate a 119-Base Pair Chondrocyte-specific Enhancer Element and to Define Active Promoter Segments in the Mouse Pro-α1(II) Collagen Gene

Krish Mukhopadhyay, Véronique Lefebvre, Guang Zhou, Silvio Garofalo, James H. Kimura and Benoit de Crombrugghe

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