Analysis of Transcription of the \textit{Col6a1} Gene in a Specific Set of Tissues Suggests a New Variant of Enhancer Region*

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The region extending from \(-5.4\) to \(-3.9\) kilobase pairs from the transcription start site of the \textit{Col6a1} gene has been previously shown to contain sequences activating tissue-specific transcription in articular cartilage, intervertebral disks, subepidermal, and vibrissae mesenchyme and peripheral nervous system (Braghetta, P., Fabbro, C., Piccolo, S., Marvulli, D., Bonaldo, P., Volpin, D., and Bressan, G. M. (1996) \textit{J. Cell Biol.} 135, 1163–1177). Analysis of expression of deletions of this region in transgenic mice has identified the 383-base pair fragment E–L as the most active sequence of the region. Linker-scanning mutagenesis analysis of segment E–J, which spans the 5′ 245 base pairs of E–L and is sufficient for high frequency expression in articular cartilage, showed that all the mutations reduced transcription considerably, suggesting that the integrity of the entire cluster of elements is necessary for enhancer activity. Electrophoretic mobility shift assays with nuclear extracts derived from various sources showed that fragment E–J binds numerous transcription factors (at least 22). These factors are present in most cells, expressing and nonexpressing \(\alpha 1(\text{VI})\) collagen mRNA, but in different relative proportions, and none of them appears to be cell type-specific. Several lines of evidence indicate that sequence elements of the enhancer may have different functional roles in various cells. The data configure the \(-5.4\)/\(-3.9\) region of the \textit{Col6a1} gene as a new type of tissue-specific enhancer, characterized by a variety of tissues supporting its activation and by the dependence of its function only on ubiquitous transcription factors. This type of enhancer is postulated to be particularly important for genes such as those of the extracellular matrix, which are often expressed with broad tissue specificity.

Genes of the extracellular matrix are very often among targets of terminal differentiation programs. In most cases, expression of the genes is the result of transcriptional regulation attained by tissue-specific enhancers. Well characterized examples are genes such as osteocalcin, collagen I, osteopontin, and bone sialoprotein in osteoblasts, and collagen II and XI in chondroblasts. The exclusive transcription of osteocalcin and the high level expression of \(\alpha 1(\text{I})\) collagen, osteopontin, and bone sialoprotein are controlled by sequences binding Osf2/\(\text{Cbfa1}\), a transcription factor necessary for the differentiation of osteoblasts \((1)\), whereas transcription of \(\alpha 1(\text{II})\) and \(\alpha 2(\text{XI})\) genes requires sequences recognized by Sox9 and other members of the high mobility group class of transcription factors, which are involved in cartilage differentiation \((2–6)\). Thus, the identification and analysis of enhancers responsible for tissue-specific expression of extracellular matrix components are important not only to understand the regulation of their genes but also to clarify the genetic control of differentiation programs.

Our group has undertaken the study of regulation of collagen VI in the mouse and has identified several sequences of the 5′-flanking region of the \textit{Col6a1} gene active in transcriptional control \((7–10)\). In particular, analyses in transgenic mice have located three regions responsible for tissue-specific transcription at high frequency \((8)\). The 0.6 kb just upstream of the transcription start site drives expression in the superficial and muscular aponeurotic system and in tendons. A second fragment, from \(-7.5\) to \(-6.2\) kb, induces transcription in joints, intervertebral disks, vibrissae, skeletal muscle, and meninges. Finally, the sequence between about \(-5.4\) to \(-3.9\) kb from the transcription start site contains information for expression in articular cartilage, intervertebral disks, nerves, vibrissae, and subepidermal mesenchyme. The strong activating capacity of the \(-5.4\) to \(-3.9\)-kb sequence has been confirmed by experiments with transgenic mice, where promoter-CAT fusions including this region are expressed in several tissues over 100 times more efficiently than constructs lacking it \((9)\). One interesting feature of the \(-5.4\)/\(-3.9\) region is that information controlling transcription in cells with different embryological origin and function, including articular chondrocytes, Schwann cells, and fibroblasts, is enclosed in a relatively small DNA fragment, 1.5 kb, a size compatible with that of a single enhancer \((11)\). The question therefore arises whether the \(-5.4\)/\(-3.9\) region contains only one enhancer dictating different tissue specificities or multiple enhancers, each one responsible for transcriptional activation in only one tissue. Such enhancers are usually formed by multiple transcription factor binding elements, including positively acting factors that are spatially localized in the organism in addition to ubiquitous ones \((12)\). Data accumulated so far on genes of the extracellular matrix conform to this condition. Studies on \(cis\)-acting sequences of collagen I genes have identified distinct enhancers for activation in cells of calcified tissues, skin, and fascial and interstitial fibroblasts. When characterized, as in bone, the active sequences have been found to bind tissue-specific nuclear factors.

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1 The abbreviations used are: kb, kilobase pair(s); CAT, chloramphenicol acetyltransferase; bp, base pair(s); X-gal, 5-bromo-4-chloro-3-indolyl-\(\beta\)-galactoside; PCR, polymerase chain reaction;
2 P. Vitale, P. Braghetta, D. Volpin, and G. M. Bressan, manuscript in preparation.

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Similarly, discrete elements recognizing tissue-specific transcription factors are necessary for high level transcription of α1(II) and α2(XI) genes in chondrocytes (2–6). In this study we have analyzed functional and structural features of the −5.4/−3.9 region. Unexpectedly, the region neither contains a unique enhancer sequence for all five tissues nor several distinct enhancers for the different tissues; rather, sequences controlling transcription in different tissues overlap extensively but do not coincide. In addition, no cell type-specific nuclear factors could be identified. These criteria identify a novel class of enhancers responsible for tissue-specific transcription.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—For an easier understanding of the results reported in this work, some of the constructs described previously were renamed. These include p5.4A(4.0–1.4)lacZ and p5.4A(4.0–1.4)CAT (8, 9), renamed p1.4A-PPlaZ and p1.4A-PfCAT.

To synthesize reporter plasmids with deletions of the −5.4/−3.9 enhancer, DNA extending from BamHI site at about −5.4 to the EcoRI site at about −3.9, identified also as segment A–P (Figs. 1 and 2), was excised from a Spel subclone of the genomic clone MG3 (17) and cloned into the Smal site of pGEM3 after blotting with Klenow enzyme. 5′- and 3′-end deletions of A–P, including segments A–G, A–K, B–P, E–P, I–P, and L–P (Figs. 1 and 2) were generated by exonuclease III (“Erase a base” kit, Promega) using the procedures recommended by the manufacturer. Segments F–H, H–M, and N–O were derived from A–P by cutting with restriction enzymes indicated in Fig. 2. Segments E–L, E–I, I–L, and E–J were generated from A–P by PCR with appropriate primer oligonucleotides. The segments were cloned in p1.4lacZ (8) upstream of the lacZ gene, producing the constructs described in Fig. 1. Some of the segments were also cloned in p1.4CAT (17) to give the corresponding CAT constructs.

For linker-scanning analysis of the E–J segment, the p1.4LSXYCAT plasmids (where X is a number comprised from 1 to 16) were synthesized. These plasmids carry 15–16-bp replacements, including a NotI restriction site, within the E–J segment and were derived by PCR-based site-directed mutagenesis using the E–J fragment cloned into pGEM7Z (Promega) as the template. The forward and reverse primers were 35–36 bp in length and comprised the replacing sequence at the 5′-end, followed by 20 bp of E–J fragment corresponding to the sequence flanking the mutated bases. PCR reactions were carried out on 100-pg pGEM7Z(E–J) as template, using the Expand High Fidelity PCR System (Roche Molecular Biochemicals) according to the protocol supplied by the manufacturer and the following cycling program: 5 min at 94°C, 35 cycles, 30 s at 94°C, 1 min annealing at 60–70°C depending on the oligonucleotides couple used, 10 min and 30 s elongation at 72°C. The PCR-amplified products were run on a 0.8% agarose gel, and after excision of the band, the DNA was extracted and purified using QIAEX II Gel Extraction Kit (Qiagen). DNA was then digested with NotI to generate compatible ends, purified by agarose gel electrophoresis, and 50 ng were ligated overnight at 16°C in the presence of 3 units of T4 DNA ligase. After transformation, clones with the mutation were isolated by NotI digestion and sequenced to confirm the introduction of the linker sequence and to verify that errors were not introduced by the PCR into the sequences of the E–J fragment. The mutated inserts were excised by digestion with Spel and HindIII and ligated into p1.4CAT digested with the same enzymes.

Trimmers of oligonucleotides spanning portions of the E–J fragment were obtained using double-stranded oligonucleotides containing the sequence described in Fig. 2 and 4 bp of complementary protruding ends appropriately chosen in the various oligonucleotides to avoid the formation of potential binding sites for transcription factors, as monitored by using resources of the Transfac transcription factor data base (18). 60 picomoles of individual oligonucleotides in 30 μl were phosphorylated with polynucleotide kinase (10 units) in 50 μl Tris–HCl, pH 8.2, 10 μM MgCl₂, 0.1 mM EDTA, 5 mM dithioerythritol, 0.1 mM spermidine, 3 mM ATP, 10 mM 2-mercaptoethanol at 37°C, and the sample incubated for 10 min at 70°C. 1 μl of 10× linker buffer (660 μM Tris–HCl, pH 7.5, 50 mM MgCl₂, 10 mM dithioerythritol, 10 mM ATP) and 1 μl (2 units) of ligase were added, and the samples were incubated at 30°C for 10 min. After treatment with phenol/chloroform and ethanol precipitation, the DNA was resuspended in 25 μl of distilled water, 8 μl of 5× T4 polymerase buffer (50 mM Tris–HCl, pH 8.5, 15 mM Na₂SO₄, 7 mM MgCl₂, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 200 μg/ml bovine serum albumin, and 0.2 mM each dNTP) and 1 μl (1 units) of T4 polymerase were added and the sample incubated for 10 min at 37°C. After addition of 1 μl (2 units) of Klenow polymerase, incubation was extended for 10 min at 37°C for 5 min at room temperature and 5 min on ice, and the enzymes were then inactivated at 70°C for 10 min. The sample was separated by electrophoresis in a 2% agarose gel and molecular species in the range from 50 to 300 bp purified using QIAEX II Gel Extraction Kit (Qiagen). The DNAs were cloned into the SmaI site of Bluescript KS⁺ using standard procedures (19). To identify clones with the correct size (3× oligonucleotide), the inserts were released by cutting with BamHI and HindIII restriction enzymes and separated in 12% polyacrylamide gels (20). The gels were fixed in 10% ethanol, 0.5% acetic acid for 10 min with shaking, incubated with 300 ml of 0.17% (w/v) AgNO₃ for 20 min, and rinsed in 300 ml of distilled water. 100 ml of developer (3% NaOH, 0.1% formaldehyde) were added, slowly swirled to disperse the cloudy precipitate that had formed, discarded, and 300 ml of the same solution added. The gel was incubated at room temperature with mild agitation until dark bands were apparent (usually within 15 min) and rinsed and stored in 5% acetic acid. The expected structure of the constructs was confirmed by sequencing. The oligonucleotide trimers were excised by BamHI and HindIII and cloned into the Spel site of pBlCT6 (20) after blunting ends of both insert and vectors with T4 polymerase and Klenow enzymes to give constructs p1.4fx3CAT to p1.4fVIIIxCAT.

**Cells**—Cells were used for RNA and chromatin structure analysis, transient transfections, and production of nuclear extracts. Established cell lines included NIH3T3 fibroblasts, C2C12 myoblasts, EL4 lymphocytes (10), MC615 (21), and RCS (22) chondroblasts, SCT-1 (23) and RN22 (24) Schwann cells. The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, with the exception of EL4 cells, which were maintained in RPMI 1640, 10% fetal calf serum, 4 mM glutamine, and 20 mM 2-mercaptoethanol. Primary cultures of sternal chondroblasts and tenden fibroblasts from 16-day-old chick embryos were prepared and grown as described (25, 26).

**Transfections and Promoter Assays**—3 × 10⁵ cells were plated into 10-cm Petri dishes and transfected the following day with the CAT constructs and the control plasmid pRSV-luciferase (10 and 2 μg, respectively) using the calcium phosphate method (27). All subsequent manipulations and assays were performed as described previously (7).

EL4 lymphocytes were transfected by electroporation. 16 × 10⁵ cells in 0.8 ml of phosphate-buffered saline containing 2 mM 2-mercaptoethanol were transferred into a 0.4 cm-path cuvette; 16 μg of CAT construct and 2 μg of control plasmid pRSV-luciferase were added, and the sample was pulsed in a Gene-Pulser apparatus (Bio-Rad) set at 320 V, 200 μF microfarads. The cells were then kept for a few minutes on ice, diluted in RPMI medium (see above), and maintained in culture for 2 days. The cells were then harvested by centrifugation and processed for CAT and luciferase assays (7).

**Generation and Analysis of Transgenic Mice—lacZ constructs were microinjected into fertilized B6D2F1 × B6D2F1 mouse oocytes, and the developing embryos were analyzed at embryonic day 14.4–16.5. Transgenic embryos were identified by dot-blot assay of DNA purified from the yolk sac, and the transgene copy number analysis and histological examination for β-galactosidase expression were carried out exactly as described (8).

**Other Assays**—Northern blotting, DNase I footprinting, identification of chromatin DNase I-hypersensitive sites, and electrophoretic mobility shift assays were performed exactly as described (7, 10).

**RESULTS**

**Analysis of Activating Properties of −5.4/−3.9 Enhancer Region in Vivo**

In order to define sequences of the −5.4/−3.9 region responsible for transcriptional activation in different tissues, transgenic mice were produced with the constructs outlined in Fig. 1a. In these constructs the lacZ gene containing a nuclear localization signal is fused to the proximal 1.4-kb 5′-flanking sequence and to different deletions of the −5.4/−3.9 region of the Col6a1 gene. The sequence of the −5.4/−3.9 elements and the position of the start and end points of the deletions are reported in Fig. 2. The region extends from the BamHI site at −5.4 kb (site A) to the EcoRI site at −3.9 (site P). Embryos were dissected usually at 15.5 days, whole mount-stained with X-gal, and the distribution of transgene-positive cells determined by histological examination of serial sections. As expected by the
presence of the proximal 1.4-kb fragment, all expressing embryos exhibited staining in superficial and muscular aponeurotic system and tendons and allows easy detection of expressing lines. Results are reported only for those tissues in which expression was previously shown to depend on the 5.4–3.9 region, but also elements comprised within L–P and/or A–E, which, when tested separately for each tissue or group of tissues. The results of the analysis of distribution of staining is summarized in Fig. 1. For only those tissues that were previously shown to require the –5.4/–3.9 region for high level and high frequency expression of the transgene, i.e. articular cartilage, peripheral nervous system, intervertebral disks, vibrissae, and subepidermal mesenchyme. A, summary of distribution of sequences of the –5.4/–3.9 region and expression in embryos determined by X-gal staining and histological examination of serial sections. All constructs contain the proximal 1.4-kb sequence of the Col6a1 gene promoter, which drives high level transcription in superficial and muscular aponeurotic system and tendons; 1.5% (2/131); and (H–M)lacZ) expressed with very low frequency (only 3 over a total of 60 lines produced). Thus, the main regulatory region enclosing only portions of the E–L region (i.e. lacZ (A–G)lacZ, (A–E)lacZ, (A–L)lacZ, (E–I)lacZ, (I–L)lacZ, (E–J)lacZ, (F–H)lacZ, (H–M)lacZ, (I–P)lacZ, (E–J)lacZ, (E–P)lacZ, (F–H)lacZ, (H–M)lacZ, and (H–M)lacZ) expressed with very low frequency (only 3 over a total of 60 lines produced). Thus, the main regulatory region for PNS is E–L. However, as B–P is the only deletion with 100% detectable inductive activity was also found in segment E–J and that integrity of this fragment is required for high levels of expression. The data indicate that distinct elements are located within E–I and I–L and that neither element reaches high frequency expression in the absence of the other, suggesting synergism. Very weak but detectable inductive activity was also found in segment L–P (2/9) and could be narrowed down to N–O. However, a fragment containing both the E–L and the L–P segments (construct E–P) was not more active than E–L alone (3/5 and 7/10, respectively). As the expression frequency of the entire –5.4/–3.9 region in articular cartilage (construct A–P) was 100%, this suggests that full induction requires not only elements located in E–L but also elements comprised within L–P and A–E, which, when tested per se, are weakly or not inductive.

**Peripheral Nervous System**—Expression in this tissue at high frequency required the presence of the entire E–L region, as found for constructs (B–P)lacZ (5/5), (E–P)lacZ (4/5), and (E–L)lacZ (5/10). Constructs not containing any portion of the E–L region did not express in this tissue. Finally, constructs enclosing only portions of the E–L region (i.e. lacZ, (A–K)lacZ, (I–P)lacZ, (E–I)lacZ, (I–P)lacZ, (E–J)lacZ, (E–P)lacZ, (F–H)lacZ, and (H–M)lacZ) expressed with very low frequency (only 3 over a total of 60 lines produced). Thus, the main regulatory region for PNS is E–L. However, as B–P is the only deletion with 100% expression frequency, full activation probably requires additional sequences comprised in either L–P and/or B–E.

**Intervertebral Disks**—The E–L region was strongly inducing, whereas its subfragment E–J was less efficient, indicating that,  

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**Fig. 1. Functional analysis of –5.4/–3.9 sequences in vivo.** a, transgenic mice were generated with promoter constructs carrying different deletions of the –5.4/–3.9 region and expression in embryos determined by X-gal staining and histological examination of serial sections. All constructs contain the proximal 1.4-kb sequence of the Col6a1 gene promoter, which drives high level transcription in superficial and muscular aponeurotic system and tendons and allows easy detection of expressing lines. Results are reported only for those tissues in which expression was previously shown to depend on the –5.4/–3.9 region. Sites comprising various deletions are indicated by capital letters (A–P) and are defined in Fig. 2. The complete –5.4/–3.9 region is coincident with fragment A–P. Data previously obtained for p1.4(A–P. The 5′-half of the E–L (fragment E–I) was much less active (2/111). Likewise, the 3′-half of E–L (segments I–L and H–M, which is only a few base pairs longer) was expressed at lower frequency (3/10 and 0/5 respectively, overall 3/15). Among subfragments derived from E–L (E–I, L–P, F–H, H–M, and E–J), only E–J was expressed with a frequency comparable to E–L itself (5/6). One conclusion coming from these results is that the main sequence necessary for transcription in articular cartilage is included in segment E–J and that integrity of this fragment is required for high levels of expression. The data indicate that distinct elements are located within E–I and I–L and that neither element reaches high frequency expression in the absence of the other, suggesting synergism. Very weak but detectable inductive activity was also found in segment L–P (1/8) and could be narrowed down to N–O. However, a fragment containing both the E–L and the L–P segments (construct E–P) was not more active than E–L alone (3/5 and 7/10, respectively). As the expression frequency of the entire –5.4/–3.9 region in articular cartilage (construct A–P) was 100%, this suggests that full induction requires not only elements located in E–L but also elements comprised within L–P and A–E, which, when tested per se, are weakly or not inductive.

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**Intervertebral Disks**—The E–L region was strongly inducing, whereas its subfragment E–J was less efficient, indicating that,
at variance with articular cartilage, elements in the J–L sequence are necessary for high frequency activation in this tissue. Unlike articular cartilage, A–E had autonomous activity, although at low frequency (1/8), in intervertebral disks. Fragment N–O was also weakly inductive in this tissue.

Vibrisseae and Subepidermal Mesenchyme—The distribution of activating regions for these two tissues was similar, although frequencies of expressing over total transgenic lines were not exactly coincident. The E–L region was strongly activating; however, the shorter E–J segment was equally effective, suggesting that sequences of the J–L region are not crucial for high frequency expression in these tissues. As for intervertebral disks, segment A–E induced β-galactosidase expression at low frequency (1/8 in the two tissues). A weak inducing region with slightly different activity in the two locations was contained in fragment N–O (3/15 and 1/15, respectively).

The main conclusion that can be drawn from these experiments is that high level expression requires the simultaneous presence of elements contained within segment E–I and I–L and that these distinct elements act in a synergistic manner. This is true for all the five tissues examined, although at different extents. The effect is strong for the peripheral nervous system in which splitting of the E–L fragment abolishes transcription completely, whereas in the other tissues the individual halves of E–L maintain a low degree of activity. A second conclusion is that, in addition to E–L, sequences with weak or no inducing activity when tested in isolation are necessary to reach the maximal enhancer activity measured for the entire −5.4/−3.9 region. It is apparent that strongly and weakly activating sequences controlling transcription in the five tissues overlap, but do not coincide (Fig. 1b), suggesting that transcriptional activation in the various tissues examined requires the cooperation of distinct but partially common sets of nuclear factor binding elements within the −5.4/−3.9 enhancer region.

Linker-scanning Mutagenesis Analysis of the E–J Region

To map the active sequences of the enhancer region better, a linker-scanning mutagenesis analysis of the E–J segment was carried out. The choice of E–J was dictated by the fact that this was the shortest segment exhibiting considerable inducing activity in vivo in four out of five tissues investigated (Fig. 1) and in transfected cell lines in vitro (Fig. 6). Although E–J was a very weak activator in the peripheral nervous system (only 1 expressing in 26 lines generated with sequences internal to the fragment, which include E–J, E–I, and F–H, Fig. 1), it contains a large portion (about 2/3) of E–L, the smallest fragment highly expressed in this tissue. The results of these experiments are reported in Fig. 3. Each one of the mutations introduced into E–J lowered significantly (at least 50%) transcription compared with either p1.4(E–J)CAT or p1.4(A–P)CAT, indicating that all sequences included elements or part of elements important for full activation. For constructs containing LS11, LS13, LS15, and LS16, CAT expression was similar to that of the enhancerless plasmid p1.4CAT, suggesting that these mutations abolished completely enhancer performance. Comparison of the corresponding sequences with those of transcription factor binding site data bases (26) identified a consensus sequence for AP1 in the fragment covered by LS11. No significant homologies with known binding sites for transcription factors were found in the sequence spanning LS13. As for LS15 and LS16, no potential sites were revealed when the individual corresponding sequences were compared; when the data base was probed with the merged sequences a C/EBP-binding site was identified at the boundary of the two segments. Thus, the loss of enhancer activity in both p1.4(LS15)CAT and p1.4(LS16)CAT might be accounted for by the mutation of a transcription factor binding site spanning part of the two mutations.

To investigate further the activating properties of elements in the E–J region, CAT constructs, in which the proximal 1.4 kb were fused with three copies of oligonucleotides I to VIII marked in Fig. 2, were transfected into NIH3T3 fibroblasts and sternal chondroblasts, and CAT activity was determined. Addition of three copies of oligonucleotides to the 1.4-kb proximal sequence was usually activating, but only weakly (≥2-fold), compared with the addition of the E–J segment (≥5-fold) (data not shown). This was also the case of oligonucleotide VIII, which included the potential AP1-binding site mutated in LS11. These results favor the idea that the integrity of the entire E–J segment is necessary for full induction; they also suggest that the enhancer function is not dependent on one in particular but rather requires cooperation of different transcription factors.

Complexity of Transcription Factors Binding to the E–J Segment

The results of the linker-scanning mutagenesis analysis suggest that the E–J segment binds a considerable number of nuclear factors. To test this prediction, protein-DNA interaction assays were performed. DNase I footprinting assays of the E–J region produced weak protections, the major of which encompassed about 90 nucleotides of the E–J fragment (footprint 1 in Fig. 4) including completely the LS4 to LS8 segments defined in linker-scanning experiments (see Fig. 3). Other protected regions were shorter and much weaker, and two examples are given in Fig. 4 (footprints 2 and 3). Although the DNase I footprinting experiments showed that a considerable portion
of the E–J region binds nuclear factors, the weak intensity of the protections prevented further analysis of the complexity of the binding transcription factors. This aspect was therefore investigated by mobility shift assays using the 12 overlapping double-stranded oligonucleotides defined in Fig. 2, which together span the entire E–J region. A representative gel shift pattern generated with nuclear extracts from chick embryo sternal chondroblasts is given in Fig. 5. Similar patterns were obtained with nuclear extracts from other cell types, and the

data are summarized in Table I. Main features of the pattern of band distribution were the absence of retarded bands specific for only one of the cell types considered and the variation of the relative intensity of bands with different cell extracts. Moreover, some bands were common to different cell types but absent in one strain of the same cell type, thus increasing the variation among cell strains. One notable example is band Xc, which, in addition to other cell types, is found in RCS, but not in sternal chondroblasts, and in tendon, but not in NIH3T3 fibroblasts. To define better the complexity of nuclear factors binding to the E–J segment, the bands were classified on the basis of their sensitivity to EDTA and heat and of the pattern of oligonucleotides inhibiting their formation in electrophoretic mobility shift assay competition experiments (Table II). The number of bands differing for at least one of these classification criteria is 22, corresponding to an estimate of the minimum number of different types of transcription factors that can bind to the E–J fragment.

**Chromatin DNase I Footprinting Assays**

Expression of promoter constructs in vivo and the different intensity of gel shift bands in various cell types (Table I) stimulate to hypothesize that elements binding transcription factors in the E–J segment have distinct regulatory relevance in different cells. To confirm this hypothesis chromatin DNase I footprinting and in vitro promoter assays were performed. The first kind of assays were carried out with the rationale that distinct assemblies of protein complexes binding to the enhancer region would imply a different organization of chromatin in the various tissues. Mouse cells employed for this analysis comprised lines derived from tissues where the 25.4/23.9 enhancer region is stimulatory such as NIH3T3 fibroblasts, MC615 chondroblasts, and the Schwann cell line SCT-1 (8, 9 and see below); all these lines expressed the α1(VI) collagen mRNA, which was particularly abundant in NIH3T3 and MC615 (Fig. 6a). The analysis was also carried out in C2C12, a cell line derived from the myogenic cell lineage, where the enhancer region is only weakly active (9). C2C12 cells produced low levels of mRNA (Fig. 6a). A final cell line included EL4 lymphocytes that did not produce detectable levels of mRNA (Fig. 6a). Four DNase I-hypersensitive sites were detected in the segment extending from 27.5 to 21.5 kb from the transcription start site (Fig. 6, b and c). As previously observed, one, indicated by * in the figure, was an invariant site present in all cells, whereas a second site, HS1, mapping at about −0.1 kb, was detectable in all cells expressing α1(VI) mRNA (10). Two other sites (HS2 and HS3) mapped at about −4.6 and −4.4 kb and were located within segments E–I and I–L, respectively (Figs. 6, b and c and Fig. 1b). These sites were absent in EL4 and very weak in C2C12 cells. On the contrary, at least one of

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**FIG. 4. Features of DNase I footprinting of the active enhancer region.** The [32P]-labeled E–I fragment (plus strand) was incubated with or without nuclear extract (80 μg from sternal chondroblasts) and the indicated amount of DNase I. The three protected sequences (marked by double arrows) have a weak character. Sequences mutated in linker-scanning experiments (see Fig. 3) are indicated by LS1–LS11. The sequence of overlapping oligonucleotides I to VIII used for gel shift assays (defined in Fig. 2) are also defined. Dots at the left label the position of a putative AP1-binding site contained in LS11.

**FIG. 5. Representative pattern of shifted bands generated with oligonucleotides I–XII spanning the E–J sequence (defined in Fig. 2).** The figure reports the results obtained with nuclear extracts from chick embryo sternal chondroblasts. Some of the band were weak with this nuclear extract; however, as summarized in Table I, they were stronger with other nuclear extracts and could therefore be unequivocally defined.
them was strong in NIH3T3, MC615, and SCT-1 cells. However, remarkable differences depending on the cell type could be noted; both sites were equally evident in NIH3T3 fibroblasts, whereas HS2 was strong and HS3 weak in MC615 chondroblasts. In SCT-1 HS3 was undetectable, and HS2 was interestingly shifted to more upstream regions by increasing DNase I concentration, due to shortening of the DNA fragment detected by the probe used (Fig. 6, b and c). These results indicate differences of chromatin structure within the enhancer region in various cell types.

**Table I**

| Band | Chick embryo sternal chondroblasts | RCS (rat chondrosarcoma) | Chick embryo tendon fibroblasts | NIH3T3 (fibroblasts) | C2C12 (myoblasts) | RN22 (rat Schwann cells) | EL4 (T-lymphoma) |
|------|-----------------------------------|--------------------------|--------------------------------|----------------------|-------------------|--------------------------|------------------|
| Ia   | +                                 | +                        | +                              | ++                   | +                 | +                        | +                |
| Ib   | +                                 | +                        | +                              | ++                   | +                 | +                        | +                |
| Ic   | +                                 | +                        | +                              | ++                   | +                 | +                        | +                |
| Id   | -                                 | -                        | -                              | -                    | -                 | -                        | -                |
| IIa  | ++                                | ++                      | ++                             | ++                   | ++                | ++                       | ++               |
| IIb  | ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| IIc  | -                                 | -                        | -                              | -                    | -                 | -                        | -                |
| IIId | ++                                | ++                      | ++                             | ++                   | ++                | ++                       | ++               |
| IIId | ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| IVa  | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| IVb  | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| IVc  | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| IVd  | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| V   | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| VIa  | ++                                | ++                      | ++                             | ++                   | ++                | ++                       | ++               |
| VIb  | ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| VIc  | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| VIId | -                                 | -                        | -                              | -                    | -                 | -                        | -                |
| VIIa | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| VIIb | ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| VIIc | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| VIIId| ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| VIIIa| +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| VIIIb| ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| VIIIc| +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| VIIIId| ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| IXa  | ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| IXb  | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| IXc  | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| IXd  | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| IXe  | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| Xa   | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| Xb   | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| Xc   | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| XId  | ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| XIIa | +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| XIIb| ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| XIIc| +                                 | +                        | +                              | +                    | +                 | +                        | +                |
| XIIId| ±                                 | ±                        | ±                              | ±                    | ±                 | ±                        | ±                |
| XIIe| +                                 | +                        | +                              | +                    | +                 | +                        | +                |

**Transient Transfections with Deletions of the −5.4/−3.9 Region**

This assay allows a quantitative evaluation of relative inducing properties of regulatory regions and is expected to be informative in our analysis aimed at establishing whether similar sequences of the enhancer have slightly different cell-specific activity. Constructs were synthesized similar to those of Fig. 1a, but containing the CAT instead of the lacZ gene, and were transfected in various cells. SCT-1 cells were poorly transfected in our experiments and were excluded. Transfections were more efficient with a rat Schwann cell line (RN22), but...
the entire enhancer region, plasmid p1.4(A–P)CAT, induced CAT activity only 2-fold compared with the enhancerless construct (p1.4CAT) (data not shown), a level too low for a reliable quantitative comparison of the activating effect of deletion constructs. C2C12 myoblasts were not used in this experiments because we had previously demonstrated that there is no sig-
ificant difference of CAT expression between p1.4(A–P)CAT and p1.4CAT (9). On the contrary, the whole enhancer region increased transcription considerably in NIH3T3, MC615, and sternal chondroblasts from chick embryos (Fig. 7). The pattern of expression of deletion constructs transfected in these cells was clearly cell type-dependent. In NIH3T3 and sternal chondroblasts the inductive properties of the entire enhancer region were as high as those of segments E–J and E–L (compare p1.4(A–P)CAT, p1.4(E–J)CAT, and p1.4(E–L)CAT with p1.4CAT). On the contrary, in MC615 cells, CAT activity produced from the deleted constructs (p1.4(E–J)CAT and p1.4(E–L)CAT) was only about one-third that from the plasmid with the whole enhancer region (p1.4(A–P)CAT). Moreover, by comparing CAT expression of p1.4(E–L)CAT with that of p1.4(E–DCAT and p1.4(I–L)CAT, it can be calculated that E–I and E–L act synergistically in NIH3T3 and sternal chondroblasts (about 2.5-fold synergism) but additively in MC615. Consistent with data in vivo (Fig. 1), the A–E and L–P segments were poorly inductive when tested in isolation; in fact, expression attained with p1.4(A–E)CAT and p1.4(L–P)CAT was not significantly different from that reached by p1.4CAT in all the cell types.

### Table II

Identification of the minimal number of transcription factors binding to the E-J segment by electrophoretic mobility shift assay

All the oligonucleotide probes were tested with nuclear extracts from both chick embryo sternal chondroblasts and C2C12 myoblasts with similar results. For oligonucleotides I and VIII, experiments were also performed with nuclear extracts from EL4 lymphocytes, as some of the bands were particularly intense using extracts from these cells (see Table I).

| Band | EDTA sensitivity | Heat sensitivity | Competitor oligonucleotide | Transcription factor (TF-#) |
|------|------------------|-----------------|---------------------------|---------------------------|
| Ia   | +                | –               | I                          | TF-1                      |
| Ib   | –                | +               | I                          | TF-2                      |
| Ic   | ±                | ±               | I, XII                     | TF-3                      |
| IIa  | +                | –               | II, III, XI                | TF-4                      |
| IIb  | +                | –               | II, III, XI                | TF-4                      |
| IIc  | +                | –               | II, III, XI                | TF-4                      |
| IIIa | +                | –               | II, III, XI                | TF-4                      |
| IIIb | +                | –               | II, III, XI                | TF-4                      |
| IIIc | +                | –               | II, III, XI                | TF-4                      |
| IIIId| ±                | ±               | II                         | TF-5                      |
| IVa  | –                | +               | IV, VI                     | TF-6                      |
| IVb  | ±                | ±               | IV, VII                    | TF-7                      |
| IVc  | ±                | ±               | IV, VII                    | TF-7                      |
| IVd  | ±                | ±               | IV, VI, VII                | TF-8                      |
| Va   | +                | +               | V                          | TF-9                      |
| Vb   | +                | +               | V                          | TF-9                      |
| Vc   | –                | –               | V                          | TF-10                     |
| VIa  | –                | +               | VI                         | TF-11                     |
| VIb  | –                | +               | IV, VI                     | TF-6                      |
| VIc  | +                | –               | IV, VI, VII                | TF-8                      |
| VId  | +                | –               | IV, VI, VII                | TF-8                      |
| VIIa | ±                | ±               | VII                        | TF-12                     |
| VIIb | ±                | ±               | VII                        | TF-12                     |
| VIIc | ±                | ±               | VII                        | TF-13                     |
| VIl  | ±                | ±               | IV, VI, VII                | TF-8                      |
| VIIa | +                | +               | VIII                       | TF-14                     |
| VIIb | +                | +               | VIII                       | TF-14                     |
| VIIc | +                | +               | VIII                       | TF-14                     |
| VIIId| –                | –               | VIII                       | TF-15                     |
| VIIle| ±                | ±               | VIII                       | TF-16                     |
| IXa  | +                | –               | IX                         | TF-17                     |
| IXb  | +                | –               | IX                         | TF-17                     |
| IXc  | ±                | ±               | IX                         | TF-17                     |
| IXd  | ±                | ±               | IX                         | TF-18                     |
| IXe  | ±                | ±               | IX                         | TF-18                     |
| Xa   | +                | +               | X                          | TF-19                     |
| Xb   | –                | +               | X                          | TF-20                     |
| Xc   | +                | –               | X                          | TF-21                     |
| XIa  | +                | –               | II, III, XI                | TF-4                      |
| XIb  | +                | –               | II, III, XI                | TF-4                      |
| XIc  | +                | –               | II, III, XI                | TF-4                      |
| XIIa | –                | –               | XII                        | TF-22                     |
| XIIb | –                | –               | XII                        | TF-22                     |
| XIIc | ±                | ±               | I                          | TF-3                      |
| XIIId| ±                | ±               | I, II, III                 | TF-3                      |
| XIIle| ±                | ±               | I, II, III                 | TF-3                      |

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\[ a \text{ EDTA was added to a concentration of 10 mM or the samples incubated at 95°C for 5 min just before the assay. The bands were either unaffected (\(-\)), decreased of intensity (\(\pm\)), or completely abolished (\(+\)) by these treatments.} \]

\[ b \text{ Bands with similar properties (EDTA, heat sensitivity, and oligonucleotide competition pattern) were assumed to be due to the same transcription factor.} \]

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**Fig. 6. Determination of α1(VI) mRNA expression and chromatin structure at the −5.4/−3.9 enhancer region in different cell lines.** 

* a. Northern blot analysis of total RNA (15 μg) from the indicated cell lines; the relative amount of α1(VI) mRNA was calculated by densitometry as a percentage of the signal observed in NIH3T3 cells and normalization with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal. b. Organization of the 5′-flanking sequence of the Col6a1 gene and positioning of the DNase I-hypersensitive sites of chromatin (HS1 to HS5 and *) deduced from the data shown in c; the horizontal arrow marks the transcription initiation site. c. Detection of DNase I-hypersensitive sites of chromatin; 5 μg of DNA extracted from nuclei treated with various amounts of DNase I were digested with SphI and analyzed by Southern blotting using probe p4 indicated in b.
This work represents the first characterization of the \(-5.4/\sim3.9\) enhancer region of the \(Col6a1\) gene. This region increases transcription in cells of five tissues (articular cartilage, intervertebral disks, peripheral nervous system, vibrissae, and subepidermal mesenchyme) differing for embryological origin, anatomical location, and specific function (8). The results show very clearly that the \(-5.4/\sim3.9\) (A–P) fragment does not contain a separate enhancer specific for each one of the five tissues controlled. Instead, high level expression in the five tissues requires sequences located in one segment of 383 bp, designated E–L. In addition, our analysis suggests that activating elements are located in the \(-5.4/\sim3.9\) (A–P) region also outside the E–L fragment. These elements have very weak or no autonomous activity, but they increase enhancer performance in \(vivo\) when linked to E–L. These elements could not be studied in detail due to their low intrinsic activity.

To identify elements responsible for transcriptional induction, linker-scanning mutagenesis experiments were performed on E–J, a 245-bp fragment representing the most active portion of E–L. This analysis could not define a limited number of discrete sequences responsible for induction; on the contrary, all mutations reduced transcription significantly. The important conclusion derived from these experiments is that multiple elements are necessary for the function of the E–L segment and that these elements must be simultaneously present for full activation. This conclusion is not contradicted by the observation that a variable degree of reduction of transcription, ranging from 50 to 100%, was detected with different mutations (Fig. 3). In fact, activation reached by multiple copies of sequences corresponding to the most effective mutations was very poor, indicating the need of the contribution of other elements. Experimental evidence suggests that cooperative interactions among elements is a main feature of the E–L enhancer; the frequency of transgene expression \(vivo\) of each half of the E–L segment, fragments E–I and I–L, is dramatically reduced compared with the entire sequence. In addition, a 2.5-fold synergism between E–I and I–L can be measured in transient transfections of NIH3T3 and sternal chondroblasts. In conclusion, the E–L enhancer should be viewed as a cluster whose full transcriptional output depends on the integrity of the entire cluster. This feature characterizes several types of enhancers, the most studied is \(\beta\)-interferon (29).

As the enhancer is active in a number of cell types and is formed by several elements, one obvious question is whether each element of the cluster has the same functional role in different cells. The data indicate that this is not the case; on the contrary, they support the idea that the contribution of elements to the enhancer output varies in different tissues. The first evidence of this comes from the \(vivo\) data. A clear example is the variation of the frequency of expression attained in the five tissues by deleting 137 bp of the 3′-part of E–L. The resulting fragment, E–J, is equally highly expressed in articular cartilage and vibrissae, whereas it is not expressed in the peripheral nervous system, and the efficiency is considerably lowered in intervertebral disks. A second evidence comes from transient transfections \(in vitro\), where a synergistic effect between E–I and I–L could be measured in NIH3T3 cells and in sternal but not MC615 chondroblasts. This result may seem surprising in view of the fact that both sternal and MC615 chondroblasts produce high levels of \(\alpha1(VI)\) mRNA (Fig. 6). It could well be, however, that regulation of mRNA expression is different in the two types of cells. The \(-5.4/\sim3.9\) region is not

\[3\] D. Girotto, C. Fabbro, P. Braghetta, P. Vitale, D. Volpin, and G. M. Bressan, unpublished results.
the only enhancer active in chondroblasts, and a distinct enhancer for these cells has been previously located at −7.5/−6.2 (8). MC615 chondroblasts are transformed cells containing an active SV40 large T antigen. This may influence the activity of the two enhancers compared with normal cells. Alternatively, high levels of mRNA in MC615 may be due to post-transcriptional regulation, resulting in stabilization of the molecule. An indirect evidence that the contribution of elements of E–L to the enhancer output may vary in different tissues comes from the analysis of protein binding to the E–J segment by gel shift assays (Tables I and II). The total number of factors binding to E–J in different cells was estimated to 22, none of which exhibited a cell type-specific distribution. Not all factors were present in each cell line, and their relative proportion was varied in different cells. Unfortunately, the weak intensity of protection in DNase I footprinting experiments did not allow us to confirm the high number of binding transcription factors using this assay coupled with oligonucleotide competition and to perform an extensive analysis of structural interactions in different cells by using the corresponding nuclear extracts. However, an indication of the different composition of protein complexes binding to the E–J region in various cells comes from DNase I footprinting of chromatin, showing that the intensity and features of the two hypersensitive sites located within E–L depend on the cell line. The formation of hypersensitive sites by transcription factors bound to DNA has been proposed to be due to either direct alteration of DNA structure (30) or to the increased probability of keeping a region nucleosome-free (31). In both hypotheses, variability of features of the hypersensitive sites are assumed to be the consequence of differences in the binding of transcription factors. The properties described delineate the E–L region of Col6a1 investigated as a new variant of tissue-specific enhancer whose main features are as follows: (i) the region controls transcription in several cell types; (ii) it comprises a large number of elements binding transcription factors with broad distribution; and (iii) high level tissue-specific transcription is achieved by cooperative contribution of sets of regulatory elements that are partially distinct in different tissues. It should be noted that tissue specificity of transcription distinguishes this type of enhancer from those, such as the interferon-β enhancer, that are dependent on ubiquitous factors and require cooperative interactions among elements (29) but whose activation is not cell type-specific (32).

If the function of the Col6a1 enhancer is based on ubiquitous nuclear factors, why is it not active in all cell types? The answer to this question will require future work, but a few possibilities will be considered here. In cells that do not express collagen VI, the Col6a1 enhancer may be inactive because the entire gene is repressed. One example may be lymphocytes, in which Col6a1 constructs, with or without the enhancer, are equally not transcribed, as indicated by the finding that CAT activity was the same as non-transfected controls. In a previous paper (10) we observed that a sequence at about −0.1 kb from the transcription start site binds AP1 in collagen VI-expressing cells but is a specific factor not related to AP1 in lymphocytes. This site corresponds to a hypersensitive site of chromatin (HS1) present in all cells expressing Col6a1 and absent in lymphocytes. We can assume that this lymphocyte-specific factor is a repressor that turns off completely transcription of the Col6a1 gene in these cells by altering the structure of chromatin close to the promoter. We are presently testing this possibility.

Different conditions may be responsible for the lack of expression of the −5.4/−3.9 enhancer in some tissues that produce collagen VI. This is the case, for example, of connective tissues associated with viscera, particularly the digestive tract and the lung, for which we have previously assumed the existence of a separate enhancer(s) (8). One hypothesis is that these tissues contain different variants or isoforms of same transcription factors binding to the −5.4/−3.9 region. Given that the interaction of elements in this enhancer region is cooperative, these variants and isoforms may not be equally effective in the assembly of a protein complex on the enhancer sequences. It has become increasingly apparent in recent years that enhancers stimulate transcription by driving the cooperative assembly of specific three-dimensional DNA-protein complexes called enhanceosomes, which increase recruitment of the transcription machinery to the basal promoter (29, 33, 34). Genes containing enhancers are transcribed at high levels only when the appropriate set of nuclear proteins is present and a specific higher order structure (enhanceosome) is assembled (35). The lack of activation of the Col6a1 enhancer examined here in viscera could be an exemplification of this principle. A second hypothesis is that some of the transcription factors must be activated by specific signal transduction pathways in order to bind to the enhancer and that these pathways are at work only in particular cells. In other words, the activation of the Col6a1 enhancer would be dependent on the so-called “cellular context,” a concept indicating the complex of signaling molecules and signal transduction pathways present in a given cell (36). The tissue-specific activation of the Col6a1 enhancer region investigated here could reflect the permissive or non-permissive properties of the cellular context.

The final definition of the number and nature of transcription factors binding to the E–L enhancer region will require cloning of their cDNA. We have undertaken this study using the one-hybrid method with constructs carrying trimers of the oligonucleotides used for gel shift assays. At present we have completed the procedure only with oligonucleotide III. This oligonucleotide gives rise to three of the major retarded bands in gel shift assays (see Fig. 5), and we had estimated a binding capacity for a minimum of two proteins (Table II). Three different nuclear factors were isolated by the one-hybrid method and shown to bind specifically oligonucleotide III in gel shift assays. One is a ubiquitous factor; the other two belong to transcription factors for which several isoforms have been described in different tissues. This may well be a lucky coincidence, but it certainly fits very well with both our appraisal of the number of binding proteins and our suggestion that the factors are either present in all cells or represent tissue variants/isoforms of widely distributed transcription factors.

The distribution of cells supporting activation of the Col6a1 enhancer is not ubiquitous but, nevertheless, includes a wide variety of tissues. Despite the broad spectrum of expressing cells, the molecular set up controlling transcription is likely to be sufficiently different in each of them to allow cell type-specific characteristics of expression. Thus, enhancers such as that studied here are expected to confer particular flexibility of expression to the genes they control. Enhancers characterized so far in genes of the extracellular matrix, such as those responsible for collagen I in calcified tissues and collagen II in cartilage, are active only in a restricted number of related cell types, and their mechanism occurs through binding of tissue-specific factors (1–6) and therefore differ from that examined in this study. However, given the widespread expression of the majority of extracellular matrix components, we expect that enhancers like that of Col6a1 studied here will be identified in many other genes.

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