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# Cell Type-specific Transcription of the α1(VI) Collagen Gene

## ROLE OF THE AP1 BINDING SITE AND OF THE CORE PROMOTER*

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**Analysis of the chromatin of different cell types has identified four DNase I-hypersensitive sites in the 5′-flanking region of the α1(VI) collagen gene, mapping at −4.6, −4.4, −2.5, and −0.1 kilobase (kb) from the RNA start site. The site at −2.5 kb was independent from, whereas the other three sites could be related to, α1(VI) mRNA expression. The site at −0.1 kb was present in cells expressing (NIH3T3 and C2C12) but absent in cells not expressing (EL4) the mRNA; the remaining two sites were apparently related with high levels of mRNA. DNase I footprinting and gel-shift assays with NIH3T3 and C2C12 nuclear extracts have located a binding site for transcription factor AP1 (activator protein 1) between nucleotides −104 and −73. When nuclear extracts from EL4 lymphocytes were used, the AP1 site-containing sequence was bound by proteins not related to AP1. The existence of the hypersensitive site at −0.1 kb may be related to the binding of AP1 and of additional factors to the core promoter (Piccolo, S., Bonaldo, P., Vitale, P., Volpin, D., and Bressan, G. M. (1995) *J. Biol. Chem.* 270, 19583–19590). The function of the AP1 binding site and of the core promoter in the transcriptional regulation of the *Col6a1* gene was investigated by expressing several promoter-reporter gene constructs in transgenic mice and in cell cultures. The results indicate that regulation of transcription of the *Col6a1* gene by different *cis*-acting elements (core promoter, AP1 binding site and enhancers) is not completely modular, but the final output depends on the specific interactions among the three elements in a defined cell type.**

Collagens are the most abundant extracellular matrix proteins of vertebrates (1). 19 types have been characterized so far, differing in structural features and tissue distribution. In addition to maintaining the structural integrity of organs, collagens endow tissues with peculiar mechanical and biological properties depending on the pattern and the levels of expression. For this reason the regulation of expression is a key issue in collagen biology. For most collagen genes, transcription is the major regulatory step, and analyses of *cis*- and *trans*-acting elements have been obtained mainly for α1(I), α2(I), and α1(II) genes (2–7 and references therein). Several types of regulatory regions necessary for high level transcription have been identified in collagen genes. As for other genes, these include the core promoter, which comprises sequence motifs usually within −40 and +40 nucleotides from the RNA start site and may or may not include a TATA box motif; the proximal upstream activating region, which extends from about −50 to −200 base pairs from the RNA start site and contains recognition sites for a subgroup of sequence-specific DNA-binding transcription factors; and enhancers, *cis*-acting DNA sequences that increase transcription in a manner that is independent of their orientation and distance relative to the RNA start site (8). Other important transcription control regions, such as the locus control region, have not been identified yet in collagen genes. The locus control region was recognized initially in the β-globin gene cluster (9) and has now been characterized in several other genes (8, 10). The locus control region is necessary to convert an inactive locus to a state competent for transcription, a condition detected by an increase in sensitivity of chromatin to digestion by DNase I. Subsequent transcription ensues by additional specific regulatory sequences, which, when active, usually introduce additional DNase I-hypersensitive sites. For example, five hypersensitive sites have been detected in the β-globin locus control region (10), and additional hypersensitive sites are located close to the core promoter of transcribed genes (9). Although, as stated above, no locus control regions have been defined yet, a correspondence between hypersensitive sites and actual transcription has been found also for collagen genes, in particular α2(I) and α1(I) (5, 11). As for the manner in which the different *cis*-acting regulatory elements contribute to the transcriptional regulation of a collagen gene, the available data suggest that they act in a modular way (4, 5, 12, 13). As proposed recently by Arnone and Davidson (14), this means that each region contributes "a particular regulatory function that is a subfraction of the overall combined regulatory function executed by the complete system" independently from the other regions. A corollary of this view is that tissue specificity of transcription is contributed by enhancers and is independent of the core promoter, whose function is the assembly of the basal transcription apparatus; hence, in experiments with transgenic animals, promoter-reporter gene constructs are expected to give rise to the same temporal and spatial pattern of expression whether using the homologous or a heterologous promoter. The few experiments addressing this issue for collagen genes confirm the above prediction (5).

We have recently undertaken a study of the regulation of transcription of the α1 chain of type VI collagen, a gene that has been linked to Bethlem myopathy in humans (15). These studies have identified several regulatory regions within the 7.5 kb[1] of 5′-flanking sequence, including the basal promoter; module(s) activating expression at low levels in tendons and at

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[1] The abbreviations used are: kb, kilobase(s); AP1, activator protein 1; CAT, chloramphenicol acetyltransferase; HS, hypersensitivity site.

high levels at the insertions of the superficial and muscular aponeurotic system within about 600 bases from the transcription start site; enhancer modules for transcription in articular cartilage, intervertebral discs, vibrissae, the peripheral nervous system, and subepidermal mesenchyme, located between about −5.4 and −4.0 kb; and region(s) stimulatory for transcription in articular cartilage, intervertebral discs, meninges, and skeletal muscle between −7.5 and −6.2 kb (12, 13, 16). In this paper we have identified several DNase I-hypersensitive sites in the 5′-flanking region of the gene. One of these sites, located at about −0.1 kb from the transcription initiation site, is detectable only in cells expressing collagen VI mRNA and contains a recognition motif for the transcription factor AP1. Analysis of the function of the AP1 site *in vitro* and *in vivo* in the context of the homologous and of a heterologous promoter indicates that both the AP1 site and the core promoter play an important role in the regulation of tissue-specific transcription of the *Col6a1* gene.

## EXPERIMENTAL PROCEDURES

*Isolation of Nuclei and Analysis of DNase I-hypersensitive Sites in the Chromatin*—NIH3T3 and C2C12 cell lines were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. EL4 lymphocytes were grown in RPMI 1640, 10% fetal calf serum, 4 mM glutamine, and 20 μM β-mercaptoethanol. Nuclei were prepared from six large plates (22.5 × 22.5 cm) of confluent NIH3T3 or C2C12 cells and from 600 ml of EL4 lymphocytes ($44 \times 10^5$ cells/ml) as described (17) with minor modifications. Adherent cells were washed extensively with phosphate-buffered saline, scraped in the same buffer using a Cell-Lifter (Costar), and harvested by centrifugation for 10 min at 400 × *g*. EL4 cells were collected by centrifugation, resuspended twice in phosphate-buffered saline, and centrifuged. The packed cell volume was measured and cells resuspended in 10 × packed cell volumes of buffer 1 (15 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.5 mM EGTA, 1.9 M sucrose, 0.1% Triton X-100, 0.5 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each pepstatin, leupeptin, and aprotinin). Cells were then lysed in a Dounce cell homogenizer with five strokes of a type B pestle, 10 packed cell volumes of buffer 2 (buffer 1 without Triton X-100) were added, and the refractive index of the suspension was adjusted to 1.40–1.42 with buffer 3 (buffer 1 without Triton X-100 and sucrose). The samples were centrifuged at 12,000 × *g* for 10 min and resuspended in 5 packed cell volumes of buffer 4 (buffer 1 lacking EDTA, EGTA, and Triton X-100 but containing 0.34 M sucrose). The absorbance of 5 μl of the suspended nuclei was measured at 260 nm. The nuclei were pelleted again at 12,000 × *g* for 10 min and resuspended in buffer 4 at an absorbance of about 0.2 $A_{260}$/ml. All manipulations were carried out at 4 °C.

For each digestion, 5 $A_{260}$ nuclei were adjusted to a volume of 80 μl with buffer 4, and 10 μl of DNase I assay buffer (400 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$) and 10 μl of DNase I (Sigma) diluted to a concentration of 0–6 units/μl were added. The samples were incubated at 37 °C and the reaction interrupted with 200 μl of stop buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS). The nuclei were then treated with 3 μl of RNase (50 μg/ml) at 37 °C for 1 h followed by the addition of 40 μl of proteinase K (20 mg/ml) at 37 °C for one night under mild agitation. The DNA was extracted by phenol/chloroform and precipitated by adding 1 volume of isopropyl alcohol and 0.1 volume of 5 M $NaClO_4$. After centrifugation, the DNA was suspended in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. 5 μg of DNA was digested with the selected restriction endonuclease and run in a 0.8% agarose gel. The DNA fragments were transferred into nylon filters (GeneScreen Plus, NEN Life Science Products) and hybridized with an appropriate $^{32}$P-labeled probe (18).

*DNA Constructs*—The cloning of fragments −82/+41 and −215/+41, which include the indicated nucleotides from the transcription start site of the *Col6a1* gene into pGEM3 vector and the fusion of the fragments into plasmid pBL6CAT to derive p82CAT and p215CAT, was described previously (16). Both plasmids contain the core promoter, which extends from nucleotides −75 to +25. To obtain pEn82CAT and pEn215CAT, the *Bam*HI-*Eco*RI fragment extending from −5.4 to −3.9 kb (19), which acts as a strong enhancer for expression in a specific set of tissues (12), was cloned into p82CAT and p215CAT upstream of the promoter region. Similar fusion constructs with the *Escherichia coli lacZ* gene replacing the CAT gene were synthesized starting from the promoterless plasmid pNSlacZ, in which the β-galactosidase sequence is fused with the nuclear localization signal of SV40. First, the −82/+41 and −215/+41 fragments were inserted into pNSlacZ to give p82lacZ and p215lacZ, and then the −5.4/−3.9 enhancer region was cloned upstream of the promoter fragments to produce pEn82lacZ and pEn215lacZ. A homologous set of CAT and lacZ constructs was also synthesized, where the human β-globin substitutes for the *Col6a1* gene promoter. The steps in the synthesis of these vectors were the release of the fragment −215/+41 from pEn215CAT or pEn215lacZ and the cloning, in its place, of a fragment from pBGZA, which contains sequences from −37 to +12 of the human β-globin gene (20), thus obtaining pEnβGCAT and pEnβGlacZ. The AP1 binding site of *Col6a1* gene was added to these plasmids by amplifying the region from −71 to −124 of p215CAT by polymerase chain reaction and ligating it between the enhancer region and the β-globin promoter. The resulting vectors were identified as pEnAP1βGCAT and pEnAP1βGlacZ. Finally, the β-globin promoter from pBGZA and the fragment containing the AP1 site fused with the β-globin promoter from pEnAP1βGCAT were cloned into pBL6CAT to give pβGCAT and pAP1βGCAT. All plasmids were purified by CsCl gradient centrifugation and sequenced to verify correct cloning.

*Generation and Analysis of Transgenic Mice—lacZ* constructs were microinjected into fertilized B6D2F1 × B6D2F1 mouse oocytes and the developing embryos analyzed at E14.0–E15.5. 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 (12).

*Promoter Assays*—NIH3T3 and C2C12 cells were grown as described above; $3 \times 10^5$ cells were plated into 10-cm Petri dishes and transfected the following day with the CAT plasmids using the calcium phosphate method (21). All subsequent manipulations and assays were performed as detailed previously (16).

*DNase I Footprinting*—The fragment −215/+41 was labeled at either end with $^{32}$P-dNTPs and Klenow enzyme and purified by agarose gel electrophoresis (22). DNase I digestion and electrophoretic analysis of the products were carried out using established protocols (16).

*Electrophoretic Mobility Shift Assay*—The synthesized double-stranded oligonucleotides used included AP1-Col6a1, which encompasses nucleotides −104/−73 of the α1(VI) chain gene promoter (19); AP1-cons (5′-AAGCATGAGTCAGACAT-3′), which contains the binding consensus sequence of AP1 (23); and AP1-mut, in which bases 10 and 11 of the previous oligonucleotide (TC) were mutated to GG. Purification of nuclear extracts from NIH3T3 and C2C12 and EL4 cells, labeling of oligonucleotides with [$^{32}$P]ATP, and assay procedures were as described (16). Antibodies against c-Fos, c-Jun, JunB, JunD, and Fra-1 for supershift experiments were purchased from Santa Cruz Biotechnology Inc., and 0.1–0.4 μl was used in each reaction.

## RESULTS

*Identification of a DNase I-hypersensitive Site Proximal to the Basal Promoter of the Col6a1 Gene*—Given the frequent association of DNase I-hypersensitive sites with regions that control transcriptional regulation (9, 10), the hypersensitive sites located within 7.5 kb of the 5′-flanking sequence of the *Col6a1* gene were identified. Because the presence of hypersensitive sites is usually related to the state of transcriptional activity of a gene in a given cell type, mapping was carried out in three cell lines that express different levels of α1(VI) mRNA. These lines include NIH3T3 fibroblasts, in which the steady-state concentration of mRNA is the highest; C2C12 myoblasts, which contain about 10-fold less mRNA; and the T cell line EL4, in which the mRNA is undetectable (data not shown). Isolated nuclei were treated with DNase I, and the purified DNA was digested with either *Sph*I or *Bam*HI and analyzed by Southern blotting. Fig. 1 shows the results obtained after digestion of DNA with *Sph*I, but similar results were observed after treatment with *Bam*HI (data not shown). In addition to the 9-kb *Sph*I-*Sph*I fragment, the probe hybridized with four other bands in NIH3T3 cells. One band of about 4 kb (labeled * in Fig. 1) was similarly present in C2C12 and EL4 cells and was therefore not related to the level of expression of the α1(VI) mRNA. The corresponding hypersensitive site, which maps at about −2.5 kb from the RNA start site, is probably caused by a region of chromatin constitutively susceptible to DNase I di-

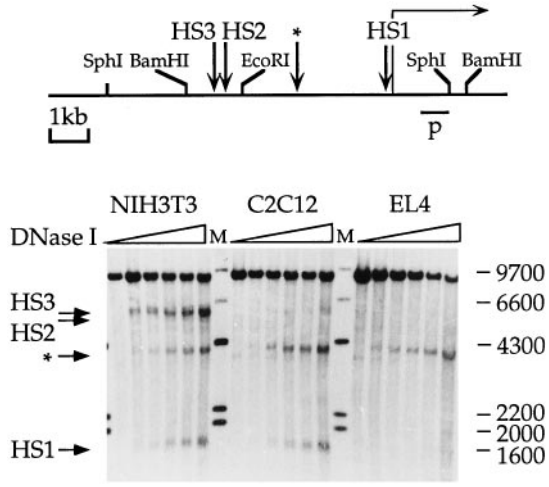


FIG. 1. **Identification of chromatin DNase I-hypersensitive sites in the 5′-flanking region of the *Col6a1* gene.** The region extends from about 7.5 kb upstream (5′-*Sph*I site) to 2 kb downstream (3′-*Bam*HI site) from the transcription start site (*horizontal arrow*). *Upper panel*, summary of the location of the hypersensitive sites found in different cell lines. The sites are indicated by *vertical arrows*. *p*, probe used for Southern blotting analysis shown in the *lower panel*. *Lower panel*, Southern blotting analysis of DNA extracted from nuclei from different cell lines, treated with various amounts of DNase I, digested with *Sph*I, and hybridized with probe p defined in the *upper panel*. * points to a hypersensitive site detectable in all cells analyzed, irrespective of the expression of α1(VI) mRNA. HS1, HS2, and HS3 label the position of hypersensitive sites present only in cells expressing the mRNA. The sites are located at −0.1, −4.4, and −4.6 kb, respectively. *M*, DNA markers.

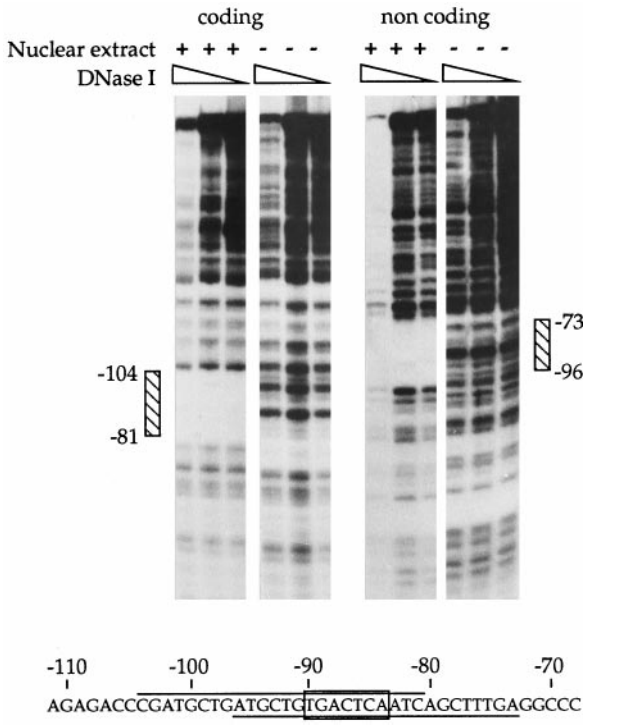


FIG. 2. **DNase I footprinting analysis of the region extending from −215 to +41 base pairs from the transcription start site.** *Upper panel*, separation of DNA fragments in denaturing 8% polyacrylamide gel. 100–20 ng and 5–0.4 ng of DNase I were added to samples with and without nuclear extract, respectively. 50 μg of nuclear extract purified from NIH3T3 cells was used in the indicated reactions. The protected sequences are indicated by *slashed boxes*. *Lower panel*, sequence of the *Col6a1* gene promoter spanning the protected region. The *lines* over and under the nucleotide sequence represent the protection of the coding and noncoding strand, respectively. The sequence of the putative AP1 binding site is *boxed*.

gestion and not dependent on the state of transcription of the gene, as described for some DNase I-hypersensitive sites in the *Col1a1* gene (11). A broad band at about 6 kb was very strong in NIH3T3 fibroblasts, very faint in C2C12 cells, and absent in EL4 lymphocytes. Rehybridization of the filter with a probe located at the 5′-end of the *Sph*I-*Sph*I fragment revealed that the hybridization signal was composed by two bands (data not shown) and was therefore marked HS2 and HS3 in Fig. 1. The characterization of these two hypersensitive sites, which map at about −4.4 and −4.6 kb and were associated with high expression of α1(VI) mRNA, will be described in a separate report.[2] Finally, the band of about 1.6 kb was distinctive of cells expressing α1(VI) mRNA because it was lacking in nonexpressing EL4 T cells. This band corresponded to a hypersensitive site located at about −0.1 kb (HS1 in Fig. 1).

*Characterization of the Region Corresponding to HS1*—The hypersensitivity of chromatin to nucleases is caused by structural features of chromatin brought about by assembly of nuclear factors at defined sequence elements (9, 10). In a previous paper we showed that several nuclear factors bind to nucleotides −75 to +8 from the RNA start site (16), a region that partially overlaps with the *Col6a1* core promoter (see "Discussion"). To locate other possible transcription factors binding sites close to the region where HS1 maps, DNase I footprinting assays were carried out with a probe spanning nucleotides −215 to +41 and nuclear extracts from NIH3T3 cells. One protected sequence was identified extending from −104 to −73 (Fig. 2, *upper panel*). The sequence contained the core motif of the binding site for transcription factor AP1 (TGAG/CTC/AA) (Fig. 2, *lower panel*) (23). Actual binding of AP1 to the protected sequence was tested by gel-shift assay, in which a probe including the AP1 site of the *Col6a1* gene gave rise to one retarded band in the presence of proteins isolated from NIH3T3 nuclei (Fig. 3*A*). The formation of the band was inhibited by the cold oligonucleotide (*lanes* labeled AP1-Col6a1 in Fig. 3*A*) and by an oligonucleotide with the consensus sequence of the AP1 binding site (22) (AP1-cons in Fig. 3*A*), but not by an oligonucleotide with a mutated version of the consensus motif (AP1-mut in Fig. 3*A*). Supershift assays with antibodies against the molecular components of AP1 factor c-Fos, Fra-1, c-Jun, JunB, and JunD revealed that the complex contained JunD (Fig. 3*B*). A retarded band with similar characteristics was detected with nuclear extracts purified from C2C12 cells (data not shown). On the contrary, the retarded bands produced by EL4 nuclear extracts with the AP1-Col6a1 probe had completely different properties: they were not competed by the AP1-cons oligonucleotide (Fig. 3*C*), and none of the antibodies mentioned above induced supershifting (data not shown). Parallel gel-shift experiments using the AP1-cons oligonucleotide as probe were also performed. These experiments established that the band retarded by incubation with NIH3T3 or C2C12 nuclear extracts was competed by both AP1-cons and AP1-Col6a1 oligonucleotides and that the band was supershifted only by antibodies against junD (data not shown). Incubation of the AP1-cons probe with EL4 nuclear proteins produced one major band that was competed by cold oligonucleotide AP1-cons and, unexpectedly also by AP1-Col6a1 (Fig. 3*D*). The band was supershifted by antibodies to fra-1 and junD (data not shown). These results suggests that the AP1 recognition site of the *Col6a1* gene has the potential to bind AP1 complexes of EL4 cells, although, as shown in Fig. 3*C*, direct binding could not be detected.

*Role of the AP1 Binding Site and of the Core Promoter in Tissue-specific Transcription in Vivo*—In previous papers we

[2] D. Girotto, P. Braghetta, C. Fabbro, P.Vitale, D. Volpin, and G. M. Bressan, in preparation.

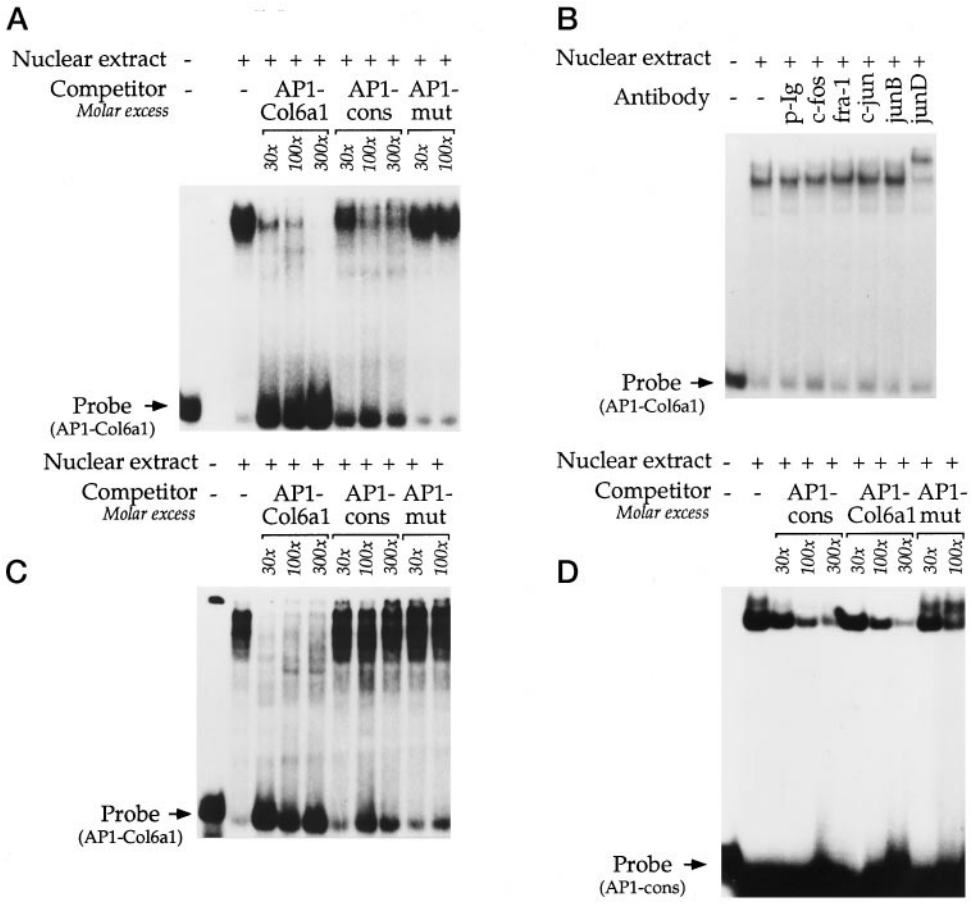


FIG. 3. **Analysis of nuclear factors binding using electrophoretic mobility shift assays.** Double-stranded oligonucleotide AP1-Col6a1, which spans the protected sequence identified by DNase I footprinting in Fig. 2, bases −104 to −73, was used as probe in *panels A–C*. Double-stranded oligonucleotide AP1-cons, which contains the binding consensus sequence of AP1 (23), was used as probe in *panel D*. 2–4 μg of nuclear extracts from NIH3T3 cells (*panels A* and *B*) or from EL4 lymphocytes (*panels C* and *D*) was employed in each reaction. Competition assays (*panels A*, *C*, and *D*) were carried out with cold oligonucleotide AP1-Col6a1 and with oligonucleotides AP1-cons and AP1-mut, which contain inactivating mutations of the consensus binding sequence for AP1. For supershift experiments (*panel B*), either preimmune Ig (*p-Ig*) or the indicated antibodies against the molecular components of AP1 were added to the reaction mixture.

have reported on transient transfections carried out with various CAT-Col6a1 promoter constructs (13, 16). A comparison of CAT expression from plasmids p215CAT and p82CAT, which contain and lack the AP1 binding site, respectively, suggested an activating role of the site. However, the same plasmids, or similar constructs carrying the *E. coli lacZ* instead of the CAT gene, were not expressed in mouse transgenic lines, so that the function of the AP1 site *in vivo* could not be determined (12, 13). To overcome this difficulty the constructs of Fig. 4*A* were designed, with the rationale that the presence of the enhancer containing region −5.4 to −3.9 (12) would overcome silencing of the basal promoter, with or without the AP1 site, *in vivo*. Moreover, to test whether or not the function of the AP1 site and of the enhancer region was dependent on the type of basal promoter, the constructs depicted in Fig. 4*B* were synthesized, in which the β-globin promoter, which contains a TATA box, replaced the core promoter of *Col6a1*, which lacks a TATA box. The four constructs were microinjected into fertilized oocytes, and β-galactosidase expression was examined in the founder transgenic embryos. The presence of the AP1 binding site increased the percentage of expressing mouse lines, and the effect was particularly relevant (3-fold) with the constructs containing the β-globin basal promoter (Fig. 4). Although the pattern of expression of the transgenes resembled that described previously (12), the histological analysis revealed interesting functional features of the AP1 binding site (Table I). The parameters considered to estimate the effect of the AP1 site

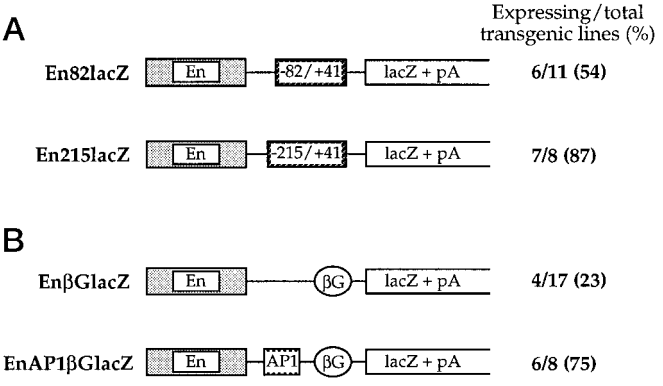


FIG. 4. **Constructs used to generate transgenic mouse lines to analyze the function of the core promoter and of the AP1 binding site of *Col6a1* gene *in vivo*.** All of the constructs include the enhancer region of the *Col6a1* gene (*En*) identified previously (12), which extends from about −5.4 to −3.9 kb from the RNA start site. Constructs in *panel A* contain sequences of the *Col6a1* promoter indicated by the *numbers*; therefore both constructs include the core promoter (nucleotides −75 to +25), whereas the AP1 binding site (nucleotides −104 to −73) is present only in En215lacZ. Constructs in *panel B* contain the human β-globin core promoter (*βG*) (nucleotides −37 to +12); EnAP1βGlacZ contains, in addition, nucleotides −124 to −73 (AP1 box) of the *Col6a1* promoter, which span the AP1 binding site. The *fractions* indicate the number of expressing over the total of transgenic mouse lines produced. The percentage is given in *parentheses*.

TABLE I

*Histological analysis of expression of different promoter-lacZ constructs in vivo*

The constructs depicted in Fig. 4 were injected into fertilized mouse oocytes, and the embryos were collected and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Dot-blot assays of the DNA purified from the yolk sacs were performed to identify transgenic embryos and to determine the transgene copy number (12). The intensity of β-galactosidase staining was evaluated by microscopic examination of serial sections on an arbitrary scale as described (12). Only tissues for which the presence of activating sequences in the 5′-flanking region of the *Col6a1* gene was previously clearly established (12) have been considered.

| Transgenic mouse line[a] | Copy no. | Tissues | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Articular cartilage | Intervertebral discs | Subepidermal mesenchyme | Vibrissae (mesenchyme) | Insertion of SMAS[b] | Tendons and ligaments | PNS[b] |
| EnβGlacZ1 | 3 | +++ | ++ | − | ++++ | − | − | +++ |
| EnβGlacZ2 | 1 | − | − | − | − | − | − | + |
| EnβGlacZ3 | 1 | ± | ± | − | − | − | ± | + |
| EnβGlacZ4 | 22 | − | − | − | − | − | ± | + |
| EnAP1βGlacZ1 | 5 | + | + | ++ | ++ | − | + | + |
| EnAP1βGlacZ2 | 3 | ++ | ++ | ± | ++ | ± | ± | +++ |
| EnAP1βGlacZ3 | 2 | ± | + | + | ++ | + | ++ | ++ |
| EnAP1βGlacZ4 | 2 | ++ | ++ | − | +++ | − | ++ | + |
| EnAP1βGlacZ5 | 2 | ++ | ++ | − | ++ | − | + | ± |
| EnAP1βGlacZ6 | 2 | ++ | ++ | − | ++ | + | ± | ++ |
| En82lacZ1 | 1 | ++ | +++ | − | − | ± | + | + |
| En82lacZ2 | 18 | ++++ | +++ | − | +++ | ± | + | +++ |
| En82lacZ3 | 1 | +++ | ++ | ± | +++ | ± | + | ++ |
| En82lacZ4 | 1 | − | − | − | − | − | − | ± |
| En82lacZ5 | 2 | ± | + | − | − | − | + | − |
| En82lacZ6 | 6 | − | − | − | − | − | ± | − |
| En215lacZ1 | 3 | ++ | +++ | − | ++ | ± | + | ++ |
| En215lacZ2 | 10 | + | + | ± | +++ | + | − | +++ |
| En215lacZ3 | 6 | + | + | ± | ++ | + | + | + |
| En215lacZ4 | 3 | +++ | ++++ | + | ++++ | +++ | +++ | ++++ |
| En215lacZ5 | 1 | +++ | ++++ | +++ | ++ | +++ | +++ | ++ |
| En215lacZ6 | 1 | ++++ | ++++ | ± | − | − | ++ | − |
| En215lacZ7 | 5 | +++ | +++ | + | ++++ | ++ | + | ++++ |

[a] The age of embryos was 14.0 days for En82lacZ4 and 15.5 days for all other lines (day of plug = 0.5).
[b] SMAS, superficial muscular and aponeurotic system; PNS, peripheral nervous system.

were the percentage of lines expressing in one particular tissue over the total of expressing lines (frequency) and the average level of expression attained in expressing lines (intensity), evaluated by the relative amount of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-positive nuclei in a defined tissue on an arbitrary scale as defined previously (12). The presence of the AP1 site was particularly important for expression at high frequency and high intensity in subepidermal mesenchyme, insertion of superficial and muscular aponeurotic system, and tendons with both the β-globin and the α1(VI) promoter. A stimulating effect of the AP1 site on frequency and intensity was also noted in articular cartilage and intervertebral discs with both promoters. The site was also required for high frequency expression in vibrissae. In the peripheral nervous system expression from the *Col6a1* gene promoter was increased slightly by the AP1; on the contrary, expression was apparently not different with or without the AP1 site for constructs carrying the β-globin promoter. The data of Table I also show that expression in different tissues was dependent on the core promoter. Thus, compared with the α1(VI) promoter, the β-globin promoter was less efficient in tendons, insertions of superficial and muscular aponeuroses, articular cartilage, and intervertebral discs; however, it induced a higher frequency of expression in the peripheral nervous system.

*Context and Cell Type Dependence of Function of the Core Promoter and of the AP1 Binding Site*—The data reported in the preceding paragraph suggest that the AP1 binding site is absolutely required for transcription in some tissues *in vivo* and that expression in different tissues changes when the core promoter is replaced by a heterologous one. However, a quantitative evaluation of the stimulatory activity of each element was not possible. In addition, the results did not allow analysis of the function of the sequences in the absence of the −5.4/−3.9 enhancer region, which was necessary for expression *in vivo*. These issues were addressed by transient promoter assays in cultured cell lines. The constructs used were similar to those described in Fig. 4 but carried the CAT instead of the *lacZ* gene. Four additional constructs lacked the upstream enhancer region −5.4/−3.9 and contained only the β-globin or the α1(VI) basal promoter with or without the AP1 site (the constructs are defined under "Experimental Procedures"). The cell cultures chosen were NIH3T3, in which DNase I HS2 and HS3 were very strong (Fig. 1), and C2C12, in which HS2 and HS3 were barely detectable (Fig. 1). The results are shown in Table II. In constructs with the β-globin promoter the AP1 site did not increase transcription in the absence of the enhancer region (compare pβGCAT with pAP1βGCAT) in both NIH3T3 and C2C12 cells. When the enhancer region was added, transcription was only slightly (2–3-fold) increased in C2C12 myoblasts with or without the AP1 site (compare pβGCAT with pEnβGCAT and pEnAP1βGCAT), suggesting that the only activating interaction was between enhancer and promoter. On the contrary, in NIH3T3 fibroblasts the enhancer region stimulated transcription about 20-fold in the absence (compare pβGCAT with pEnβGCAT) and 80-fold in the presence (compare pβGCAT with pEnAP1βGCAT) of the AP1 site. In this case the mutual interactions among the AP1 binding site, the enhancer region, and the β-globin promoter can be defined as synergistic, because transcription elements synergize when their combination produces a transcriptional rate that is greater than the sum of the effects produced by individual elements. In our experiments the amount of transcription reached in the presence of the three elements was 3.5-fold greater (fold synergism) than the sum of the effects produced when the β-globin promoter was combined separately with either the AP1 site or the enhancer region. The results were completely different with constructs containing the basal promoter of the *Col6a1* gene. Transcription from enhancerless constructs was stimulated about 5–6-fold in both NIH3T3 and C2C12 cells by the AP1 site (compare p82CAT with p215CAT). The enhancer region increased transcription 7-fold in C2C12 myoblasts (compare p82CAT and pEn82CAT), and the pres-

TABLE II
*Role of core promoters, AP1 site, and −5.4/−3.9 enhancer region on transcription in different cell types*

| Cells and construct | CAT activity[a] | Fold induction[b] | Significance[c] | Comment: type of interaction between regulatory elements[d] |
|---|---|---|---|---|
| C2C12 | | | | |
| pβGCAT | 2.2 ± 0.6 | 1 | | Single: enhancer and core promoter |
| pAP1βGCAT | 3.2 ± 1.2 | 1.4 | $p > 0.1$ | |
| pEnβGCAT | 6.8 ± 2.3 | 3.1 | | |
| pEnAP1βGCAT | 5.5 ± 0.9 | 2.5 | $p > 0.4$ | |
| p82CAT | 8.0 ± 2.9 | 1 | | Additive: AP1 site and core |
| p215CAT | 38.9 ± 13.5 | 4.9 | $p < 0.005$ | promoter; enhancer and core |
| pEn82CAT | 55.9 ± 11.0 | 7.0 | | promoter |
| pEn215CAT | 100 ± 14.4 | 12.5 | $p < 0.004$ | |
| NIH3T3 | | | | |
| pβGCAT | 1.0 ± 0.3 | 1 | | Synergistic (=3.5-fold): AP1 site |
| pAP1βGCAT | 1.2 ± 0.6 | 1.2 | $p > 0.5$ | and core promoter; enhancer and |
| pEnβGCAT | 22.7 ± 2.5 | 22.7 | | core promoter; enhancer and AP1 |
| pEnAP1βGCAT | 83.7 ± 29.1 | 83.7 | $p < 0.01$ | site |
| p82CAT | 8.3 ± 4.0 | 1 | | Possibly competitive: enhancer and |
| p215CAT | 56.0 ± 8.9 | 6.7 | $p < 0.0001$ | AP1 site interaction inhibits AP1 |
| pEn82CAT | 92.5 ± 27.9 | 11.1 | | site and core promoter |
| pEn215CAT | 100 ± 10.7 | 12.0 | $p > 0.5$ | interaction |

[a] CAT activity of individual constructs is expressed as a percent of that obtained with the pEn215CAT construct. The data represent the mean ± S.D. derived from at least four samples obtained in two separate experiments.
[b] Fold induction was calculated assuming as the unit the CAT activity of the construct containing only the core promoter.
[c] Student's *t* test was used to compare CAT activity expressed by constructs having the same design and differing only for the presence or absence of the AP1 binding site. This comparison allows the evaluation of the contribution of the AP1 binding site to the transcriptional activation in different core promoter-enhancer contexts.
[d] The type of interaction was deduced by comparing the CAT activity of constructs with the same core promoter with or without the AP1 binding site and/or the enhancer region and considering the existence of a positive interaction between the regulatory elements only when the expression from constructs containing or lacking the activating element was statistically significant. See "Discussion" for definition of various types of interactions.

ence of both elements, AP1 site and enhancer region, resulted in an additive stimulation of about 12-fold (compare p82CAT with pEn215CAT). On the other hand, expression of pEn82CAT and pEn215CAT was similar in NIH3T3 fibroblasts, suggesting that the stimulating function of the AP1 binding site was abolished in the presence of the −5.4/−3.9 enhancer region in these cells.

## DISCUSSION

The results described in this paper contribute substantial information on the function of the proximal promoter region of the *Col6a1* gene. A DNase I-hypersensitive site (identified as HS1) was localized in the chromatin at about −0.1 kb from the transcription initiation site. HS1 was detectable only in cell lines that express α1(VI) collagen mRNA, suggesting that a rearrangement of the chromatin structure in the proximal 5′-region is a necessary condition for transcriptional activation of the gene. The analysis of DNase I-hypersensitive sites also suggested that distinct levels of expression in different cell types were achieved by additional rearrangements of the chromatin at other sites. Thus, high amounts of mRNA were detected in NIH3T3 fibroblasts, where three hypersensitive sites were easily detectable at −4.6, −4.4, and −0.1 kb, whereas 10-fold lower levels of α1(VI) mRNA were found in C2C12 myoblasts, where the site at −0.1 kb was strongly and the other two sites very weakly sensitive to DNase I. Regions containing a defined set of DNase I-hypersensitive sites in chromatin are usually required for position-independent transcription of transgenes *in vivo* (9, 10). When tested alone, sequences corresponding to HS1 were completely inadequate to overcome the constraints of chromatin structure. On the other hand, they improved the function of other sites, as indicated by the relative increase of mouse transgenic lines expressing the *lacZ* reporter gene (from 54 to 87% for constructs of Fig. 4A and from 23 to 75% for those of Fig. 4B) when the AP1 binding site was present. As indicated by the high percentage of expressing lines in Fig. 4, hypersensitive sites HS2 and HS3 are very efficient in making chromatin transcriptionally competent at the site of insertion of transgenes. However, the data also point out that the hypersensitive sites detected were not sufficient for complete independence of transgene expression from the insertion site. Therefore, additional regulatory sequences and DNase I-hypersensitive sites should be identified to understand fully the transcriptional regulation of the *Col6a1* gene.

DNase I footprinting and band-shift assays have located a recognition site for transcription factor AP1 at −104 to −73 base pairs, close to where HS1 maps, suggesting that this site and probably the GA box-containing sequences identified previously between −75 and +8 (16) play an important role in determining DNase I hypersensitivity of chromatin. An AP1 binding site proximal to the basal promoter is a conserved feature of the *Col6a1* gene since the site has been found also in chicken and in human (24, 25). In addition, a similar element was recognized in the chicken α2(VI) collagen gene (26), suggesting that an AP1 binding site may be a key element in the regulation of collagen VI genes. In NIH3T3 and C2C12 cells, which express the α1(VI) mRNA, the site was actually bound by an AP1 factor complex containing JunD. In contrast, in nuclear extracts from EL4 cells, which do not express the α1(VI) mRNA, the same sequence was recognized by factor(s) not related to AP1, although the cells contain various molecular forms of the AP1 transcription factor. An obvious speculation stimulated by these results is that the presence or absence of DNase I HS1 may be determined by the difference of nuclear factor binding at sequences including the AP1 site. One possibility is that the AP1 factors of EL4 lymphocytes bind with low affinity to the *Col6a1* gene promoter, whereas the molecular form(s) present in NIH3T3 and C2C12 cells have high affinity for the site. Differences in the molecular composition of AP1 binding to distinct promoters have already been observed in various cell types (27). Alternatively, EL4 cells might contain peculiar transcription factors that are absent in the other cells and compete with AP1 protein for binding to the site. Future studies will elucidate this issue.

Analysis of transgenic mice carrying promoter-*lacZ* constructs has shown that the frequency of expressing lines and the average level of expression in the lines are variously af-

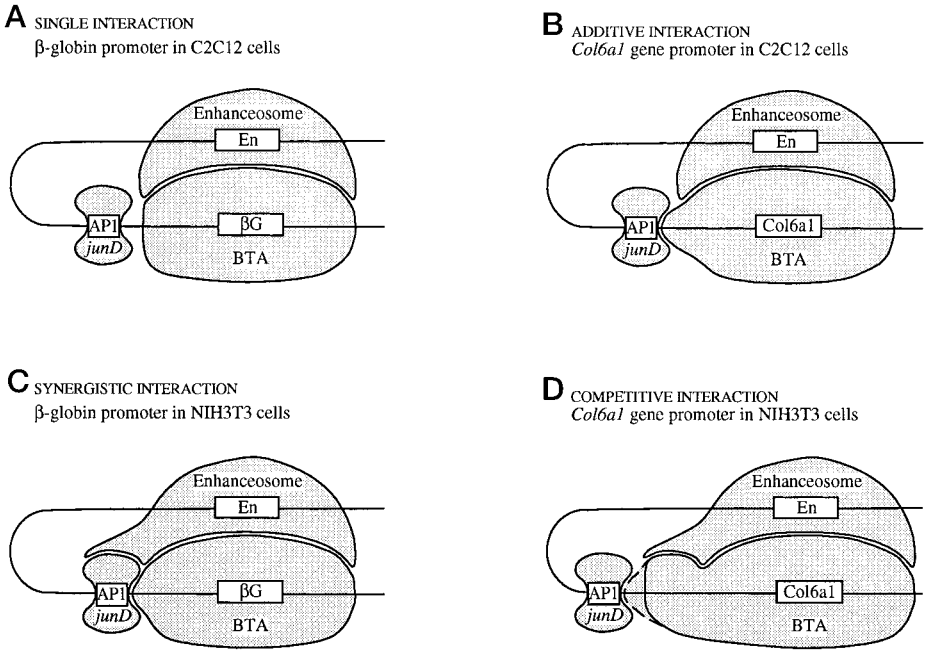


FIG. 5. Representation of different types of core promoter, enhancer region, and AP1 binding site interactions identified in transfection experiments with different cells. The three regulatory elements are bound by specific protein complexes: the core promoter, either from β-globin (*βG*) or from the *Col6a1* gene (Col6a1), is depicted in association with the basal transcription apparatus (*BTA*); the AP1 binding site is occupied by a molecular form of the AP1 transcription factor containing JunD; the enhancer region from −5.4 to −3.9 of the *Col6a1* gene (*En*) is hypothesized to bind a cell type-specific enhanceosome (33). In *panel D* the two mutually exclusive interactions of the BTA are represented: when the enhancer region is inactive or absent, the AP1 factor binds to the BTA (*dashed line*); this interaction is disrupted when an active enhancer region binds to the BTA (*solid line*). For definition of various types of interactions, see "Discussion" and Table II.

fected by the AP1 binding site in different tissues. Both parameters are particularly dependent on the presence of the AP1 site in subepidermal mesenchyme, at the insertion of the superficial muscular and aponeurotic system, and in tendons. The frequency parameter can be attributed to the capacity of a *cis*-acting region to make chromatin accessible to the transcriptional machinery, indicating that AP1 has an important structural role in these tissues. This function of the AP1 site is clearly evident also in vibrissae, where the frequency, but not the intensity, was greatly stimulated. The level of expression of a transgene probably depends on the activating potential of the *cis*-acting elements, *i.e.* the ability of the factors binding to DNA modules to recruit the transcription preinitiation complex (8). Our data lead us to conclude that AP1 is a strong activator of transcription in cells of subepidermal mesenchyme, at insertions of the superficial muscular and aponeurotic system, and tendons. On the contrary, the AP1 site does influence only marginally both the frequency and the intensity of expression of transgenes in cells of the peripheral nervous system. To explain the independence of frequency from the AP1 site it may be hypothesized that, in the peripheral nervous system, either the function of the site is replaced by another site not active, and hence not detected, in the cell cultures we have used, or opening up of chromatin is almost completely dependent on the upstream enhancer region. An intermediate situation is apparent in the remaining tissues, articular cartilage and intervertebral discs, where the AP1 site increases to some extent the frequency and intensity of expression. The *in vivo* data also suggest a role for the core promoter in tissue-specific transcriptional regulation of the *Col6a1* gene, in a way similar to that of the AP1 binding site. In fact, expression of transgenes in tendons and at the insertions of superficial muscular and aponeurotic system was more evident with the *Col6a1* promoter than with the β-globin promoter. Conversely, the frequency of expression in the peripheral nervous system was higher with the β-globin promoter. The core promoter of the *Col6a1* gene was partially characterized in previous work and was shown to exhibit several unusual features among the TATA-less promoters (16, 19). The RNA start sites are spread on a sequence of more than 70 base pairs, and the most upstream site has been denoted as +1. The major transcription initiation site is at base +21 and a second strong site at base +9. These sites resemble, but do not match exactly the consensus sequence proposed for the initiator element (+21 site: 8Py C $A_{+1}$ G C 3Py; +9 site: 9Py $G_{+1}$ G C T 8Py; consensus sequence for initiator: Py Py $A_{+1}$ N T/A Py Py; where Py indicates a pyrimidine) (28). Because it has been noted that a large number of pyrimidines surrounding the start site can impart low levels of initiator activity in the absence of either the A at +1 or the T at +3 (29), it is very likely that the sequences around +21 and +9 constitute weak initiators. These initiator elements, however, do not drive transcription unless they are linked to an upstream sequence, containing repeated GA boxes, which extends from −75 to +8 (16). This region has intrinsic promoter activity, as suggested by the observation that the fragment −82 to +41 is equally active in both orientations,[3] a property not shared by initiators (28). Considering both the putative initiator sites and the GA box-rich region, the core promoter of the *Col6a1* gene extends from −75 to +25, a sequence that closely corresponds to that used to synthesize our *Col6a1* core promoter constructs (−82/+41).

In a previous report we located the region inducing transcription in tendons and at the insertions of the superficial muscular and aponeurotic system within 0.6 kb upstream from the RNA start site (12). The new results point at the AP1 binding site as an important element contributing to activation of transcription in these tissues. In the same paper, the modules responsible for transcription in the subepidermal mesenchyme were assigned to the −5.4/−3.9 enhancer region. The present data show that expression in this tissue is strongly dependent on the homologous promoter and on the presence of the AP1 binding site. Thus, it may be speculated that transcription in the subepidermal mesenchyme requires a synergistic action of the three regulatory elements: the core promoter, the AP1 site, and the enhancer region. The overall message coming from the *in vivo* experiments is, therefore, that transcription in different tissues depends on a peculiar interplay among the three regulatory elements.

The complexity of the mechanisms of tissue-specific regulation of the *Col6a1* gene observed *in vivo* was defined further in transfections *in vitro*. The quantitative analysis of the results leads to a conclusion similar to that of the *in vivo* data: the

[3] S. Piccolo, unpublished observations.

levels and the features of transcriptional activation in different cell types depend on the specific interactions among the core promoter, the proximal activating region, and the enhancer region. Four distinct types of interaction could be identified by the data reported in Table II, as outlined in Fig. 5. In C2C12 cells the AP1 site did not interact positively with the −3.9/−5.4 kb enhancer region (Fig. 5, *A* and *B*). When the β-globin promoter was used, the only interaction was between the promoter and the enhancer (Fig. 5*A*). On the other hand, the homologous promoter was stimulated by both the AP1 site and the enhancer, and the final induction of transcription achieved with the three modules together was the sum of those obtained from the separate combinations of the promoter with the other modules (Fig. 5*B*). A completely different situation was apparent in NIH3T3 cells. The use of the β-globin promoter resulted in a synergistic activation of about 3.5-fold when all of the modules were present. The synergism can be explained by assuming that the protein complex assembled at each module interacted positively at the same time with those brought together by the other modules as indicated in Fig. 5*C*. By replacing the TATA-containing β-globin promoter with the TATA-less promoter of the *Col6a1* gene, synergism did not take place, and a fourth type of interaction of modules was observed, tentatively identified as competitive (Fig. 5*D*). Namely, although the AP1 site stimulated considerable transcription from the promoter, expression in the presence of the enhancer was similar with or without the AP1 site. This condition can be accounted for by hypothesizing that an activating interaction takes place between the homologous promoter and the AP1 site if the enhancer is inactive (or absent) and that this interaction is disrupted when the enhancer is turned on and binds to the core promoter.

The model of Fig. 5 differs considerably from the view of DNA regulatory elements acting in a modular way to control transcription deduced from studies on expression of transgenes *in vivo* by several authors including ourselves (2–5, 12, 14). The modularity of the function of *cis*-acting elements in these reports only applies to the fact that, for genes expressed in several tissues, such as most collagens, different enhancer regions activate transcription in specific subsets of tissues. A closer look at the function of the regions involved, however, shows the existence of more complex interactions among regulatory elements, which may explain peculiar features of a gene's regulation. One example is enhancer-promoter selectivity, in which the activation of only one of multiple promoters by a nearby enhancer depends on cognate interactions between the two elements (8, 30). As for our experiments, these results provide evidence that different core promoters possess distinct regulatory activities. The model of Fig. 5 is also consistent with the present knowledge on the molecular mechanisms of transcription activation, in which the final result is the consequence of specific interactions of protein complexes bound to different *cis*-acting regulatory elements. In a simplified view, the core promoter associates with the general transcription factors (31), whereas activators are bound at proximal activating sequences or at enhancers. Enhancers are usually made of specific clusters of binding sites for nuclear factors, which impose a precise alignment of the proteins on the DNA, resulting in the formation of a stable, highly stereospecific three-dimensional nucleoprotein complex called enhanceosome (32, 33). The interaction between the general transcription factors and the enhanceosome (or single activators at isolated binding sites) then determines the recruitment of the RNA polymerase II holoenzyme and the formation of a stable preinitiation complex (33, 34). It is clearly apparent from this model that any change in the composition of the three types of protein complexes (general transcription factors, enhanceosome, and activators bound at the proximal activating region) could influence RNA polymerase II recruitment. The molecular analysis of the interactions among the *cis*-acting regulatory regions of the *Col6a1* gene in different cell types will require the delineation of binding of general transcription factors to the core promoter and the characterization of the protein complex assembled at enhancer regions. These studies are presently in progress.

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