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Regulation of integrin α10 expression in chondrocytes by the transcription factors AP-2ε and Ets-1

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Abstract

Expression of integrin α10 is initiated at the beginning of chondrogenesis and continues throughout cartilage development in adult cartilage. In our study, we aim to identify regulatory sequences that control the cell-type specific expression of the human integrin α10 gene. Therefore, promoter constructs harboring 1139 bp 5′ of the transcriptional start site of the human integrin α10 gene were analyzed. Our experiments localized a promoter region that directs high levels of expression specifically in chondrocytes. A sequence analysis detected three consensus AP-2 binding sites within this functional domain. Functionality of these sites was tested and confirmed by cotransfection of AP-2 in a luciferase reporter assay. Interestingly, EMSA identified AP-2ε as the major AP-2 protein binding to the AP-2 consensus sequences. Additionally, Ets-1 was shown to be a positive regulator of the integrin α10 expression whereas Sox9 was irrelevant. Taken together, these results suggest that AP-2ε and Ets-1 are involved in the regulation of integrin α10 transcription in chondrocytes.

Keywords: Integrin; Cartilage; Chondrocytes; AP-2ε; Transcriptional regulator; Ets-1

Integrins represent a family of heterodimeric proteins, composed of an alpha and a beta subunits. There are at least 25 distinct integrins which are formed by 8 beta subunits that can combine with 18 alpha subunits. The α10 subunit interacts with the β1 subunit and binds collagen type II [1]. Immunohistochemical analysis of integrin α10 in 3-day-old mice demonstrated expression in the hyaline cartilage of joints, vertebral column, trachea, and bronchi [2]. Expression of integrin α10 appeared at embryonic day 11.5 at the same time as chondrogenesis started. After birth, integrin α10 was prominent both at the articular surface and in the growth plate [2]. Mice carrying a constitutive deletion of the α10 integrin gene had a normal lifespan and were fertile but developed a growth retardation of the long bones [3]. Analysis of the skeleton revealed defects in the growth plate after birth characterized by a disturbed columnar arrangement of chondrocytes, abnormal chondrocyte shape, and reduced chondrocyte proliferation. These results demonstrate that integrin α10 β1 plays an important role in growth plate morphogenesis and function.

Eukaryotic gene expression is subjected to the combined action of multiple DNA-binding proteins interacting with specific DNA motifs present in the promoters and enhancers of genes. The transcription factor AP-2 recognizes the palindromic sequence 5′-GCCNNNGGC-3′ [4]. AP-2 has been shown to play a crucial role in the control of gene expression in response to cell differentiation signals within neural crest and epidermal cell lineages [5]. So far, five isoforms of the AP-2 gene have been identified from humans and mice that recognize an equal binding motif [6–11]. The murine AP-2α gene was disrupted by two independent groups by homologous recombination [12,13]. The AP-2α null mice died perinatally with cranio-abdominoschisis

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and severe dismorphogenesis of the face and skull, suggesting an AP-2 effect on the skeletal development.

The *ets-1* gene was first characterized as the cellular proto-oncogene of the retroviral *v-ets* oncogene of the avian leukemia retrovirus E26. *Ets-1* is the prototype of the *ets* gene family of transcription factors (for reviews see [14–16]). The members of the Ets family are characterized by a conserved ETS domain, which binds to double-stranded DNA containing a GGAA/T core sequence. Several lines of evidence suggest that Ets-1 plays an important role in differentiation, proliferation, angiogenesis, apoptosis, and also in tumor vascularization and invasion [14,17]. Further, during embryogenesis Ets-1 mRNA expression is transiently induced during emigration of neural crest cells from which head chondrocytes derive [18].

The HMG-box-containing transcription factor, Sox9 which binds and activates a chondrocyte-specific enhancer element, is required for chondrocyte differentiation and for expression of a series of chondrocyte-specific marker genes including Col2a1, Col9a2, Col11a2, and aggrecan [19,20].

As recent studies have shown that the human integrin α10 is strongly and almost specifically expressed in chondrocytes, we aimed to determine the mode of regulation by analyzing the integrin α10 promoter. In the present study, we evaluated the functional regulatory domains in the human integrin α10 promoter by transient transfection into primary chondrocytes and a chondrosarcoma cell line and demonstrated that AP-2, Ets-1, and Sox9 binding motives may be regulatory domains for chondrocyte-specific promoter activity.

Materials and methods

Cell culture. The chondrosarcoma cell line SW1353 was obtained from the American Type Culture Collection (ATCC#HTB-94). Additionally, the colon carcinoma cell line SW480 (ATCC#CCL-228) was used. Cells were maintained in DMEM high glucose supplemented with penicillin (400 U/ml), streptomycine (50 μg/ml), L-glutamine (300 μg/ml), and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and split at a 1:5 ratio every 3 days. Primary chondrocytes were obtained from Cambrex and cultured as suggested by the manufacturer.

RNA isolation, reverse transcription, and PCR. Total cellular RNA was isolated from cultured cells or from tissues using the RNeasy kit (QIAGEN, Hilden, Germany) and cDNAs were generated as previously described [21]. PCR analysis of integrin α10 expression was performed using specific primers (*α10*-forward: 5′-CATGAGGTTCACCGCATC ACT-3′ and *α10*-reverse: 5′-AAGGCAAAGGTCACAGTCAAGG-3′ (192 bp fragment)) in a 50 μl reaction volume containing 5 μl 10× Taq-buffer, 1 μl of cDNA, 0.5 μl of each primer (20 mM), 0.5 μl of dNTPs (10 mM), 1 U of Taq polymerase, and 41.5 μl of water. The amplification reactions were performed by 36 cycles of 1 min at 94 °C, 1 min at 64 °C, and a final extension step at 72 °C for 1.5 min. The PCR products were resolved on 1.5% agarose gels.

PCR analysis for collagen II, aggrecan, MIA, and AP-2ε were performed similar by using specific primers (*collagen II*-forward: 5′-TCAACA ACCAGATTGAGAGCATCCGC-3′ and -reverse: 5′-GATTGGGGTA GACGCAAGTCTCGCC-3′ (annealing temperature 64 °C), *aggrecan*-forward: 5′-CAACAATGCCCAAGACTACCAGTGG-3′ and -reverse: 5′-GTCTGCGTTTGTAGGTGGTGGCTG-3′ (annealing temperature 58 °C), *MIA*-forward: CATGCATGCGGTCCTATGCCCAAGCTG-3′ and -reverse: 5′-GATAAGCTTTCACTGGCAGTAGAAATC-3′ (annealing temperature 60 °C), *AP-2epsilon*-forward: 5′-GAAATAGGGACTTA GCTCTTGG-3′ and -reverse: 5′-CCAAGCCAGATCCCCAACTCTG-3′ (annealing temperature 59 °C)).

Immunohistochemical staining. Paraffin sections of cartilage were screened for AP-2ε protein expression by immunohistochemistry. The tissues were fixed and subsequently incubated with primary AP-2ε antiserum (1:200) overnight at 4 °C. The secondary antibody (biotin-labeled anti-rabbit, DAKO, Germany) was incubated for 30 min at room temperature, followed by incubation with streptavidin-POD (DAKO) for 30 min. Antibody binding was visualized using AEC-solution (DAKO). Finally, the tissues were counterstained by hemalaun solution (DAKO).

Plasmid constructs. To construct the integrin α10 promoter 5′-deletion constructs, the human genomic region was amplified by polymerase chain reaction (PCR) with a 3′-reverse primer (5′-GACAAGCTTGCCTGATC GGTTTCTGTC-3′) that bound at −3 relative to the integrin α10 translational start site in conjunction with different 5′-forward primers that bound at varying distances within the upstream flanking sequence (−1139: 5′-GACGCTAGCACCTAGCTGAGGAGTTGG-3′, −960: 5′-GACGC TAGCGGAGCTGTGTCTTCACAAG-3′, −350: 5′-GACGCTAGCGA ATCCATCTCCCACTCC-3′). Subcloning of the amplified fragments into the luciferase expression vector pGL3-basic was performed via *Hin*dIII/*Nhe*I sites. The nomenclature used for each deletion construct (−1139, −960, and −350) indicates the number of base pairs of the upstream 5′-flanking sequence with respect to the ATG translation start codon.

Transient transfection and luciferase assay. DNA transfection of the SW1353 and SW480 cells was performed using Lipofectamin plus (Invitrogen, Carlsbad, CA). Primary chondrocytes were transfected by electroporation (Amaxa, Cologne, Germany). Briefly, the cells were cultured in 6-well plates using 0.2 μg of the luciferase reporter plasmid and 0.1 μg of the internal control plasmid pRL-TK each well. The cells were harvested 24 h later and the lysate was analyzed for luciferase activity (Dual-luciferase assay reagent, Promega). At least three independent transfection experiments were carried out for each construct.

Preparation of nuclear extracts. Nuclear extracts were prepared from the cultured cells by the method of Dignam et al. [22].

Electrophoretic mobility shift assay (EMSA). The EMSA was based on the binding of nuclear AP-2 to a ^{32}P-labeled AP-2 consensus oligonucleotide. Three double-stranded oligomeric binding sites for AP-2, specific for the integrin α10 promoter (AP-2-I: 5′-GTTTTCCTTGGTCTGGGG CTCCCCACAGTTC-3′, AP-2-I: 5′-GTTCAAGACCAGCCTGGCCA ACATGG-3′, AP-2-III: 5′-GGGAGGCCGAGGCAGGTGGATCTC-3′) were generated. The fragments for AP-2 correspond to three integrin α10 regions from −174 to −144 (AP-2-I), from −762 to −737 (AP-2-II), and from −798 to −775 (AP-2-III) in the upstream region of the ATG. The fragments were end-labeled and shifts were performed as described previously [21]. For the competition studies, the cold oligonucleotides were added at a 400-fold molar excess. For the antibody interference experiments, the anti-AP-2α antibody (Santa Cruz Heidelberg, Germany) and an anti-AP-2ε antiserum [9] were used. The AP-2α antibody from Santa Cruz Biotechnology reacts with AP-2α and AP-2β but not with AP-2γ, AP-2δ or AP-2ε. The AP-2ε antiserum is specific for AP-2ε [9].

Statistical analysis. Results are expressed as means ± SD (range) or percent. Comparison between groups was made using the Student's paired *t*-test. A p value < 0.05 was considered statistically significant. All calculations were performed using the GraphPad Prism software (GraphPad software Inc, San Diego, USA).

Results

Integrin α10 promoter is active in chondrocytes

One thousand one-hundred and thirty-nine basepairs of the 5′-flanking sequence of the human integrin α10 gene were fused to the promoterless luciferase reporter plasmid pGL3-basic and tested for its ability to generate luciferase activity in the transiently transfected cells (Fig. 1). As

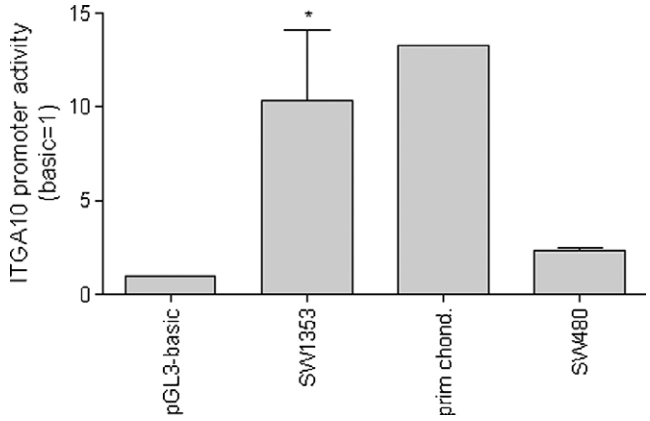

Fig. 1. Activity of the integrin α10 promoter in cells of different origin. 1139 bp of the integrin α10 promoter region was cloned into pGL3-basic and promoter activity was analyzed in SW1353, primary chondrocytes and SW480. pGL3-basic is set as one for each individual cell type. $*p < 0.05$.

expected, the construct generated high luciferase activity in SW1353 and primary chondrocytes and only very weak expression in SW480 colon carcinoma cells. This suggests that this upstream sequence of the integrin α10 gene contains elements necessary for chondrocyte-specific activity. Subsequent experiments were performed using SW1353 cells as representative for primary chondrocytes due to higher transfection efficiency.

Deletion analysis of the integrin α10 promoter

The human chondrosarcoma cell line SW1353 maintains its cartilage phenotype and expresses collagen type II, MIA (CD-RAP), and aggrecan. The synthesis of type II collagen, aggrecan, MIA (CD-RAP), and integrin α10 mRNA by SW1353 cells was confirmed by RT-PCR analysis (Fig. 2). To identify the *cis*-acting elements that drive the expression of the human integrin α10 gene in chondrocytes, we transfected a series of 5′-nested deleted promoter constructs (−350, −960, and −1139) into SW1353 cells and measured the luciferase activities generated in these cells.

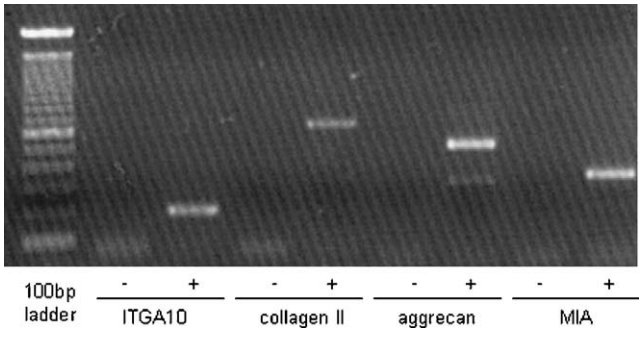

Fig. 2. Expression of integrin α10, collagen type II, aggrecan, and MIA (CD-RAP) by SW1353 chondrosarcoma cells. RT-PCR to measure the amount of expressed integrin α10, collagen type II, aggrecan, and MIA (CD-RAP) mRNA in SW1353 chondrosarcoma cells was performed to confirm chondrocytic differentiation.

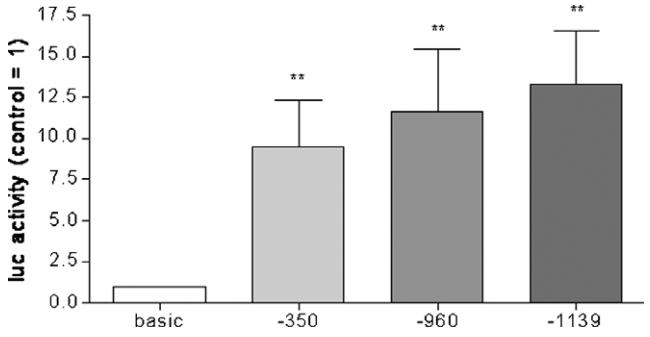

Fig. 3. Activity of fragments of the integrin α10 promoter in SW1353 cells. 350, 960, and 1139 bp of the integrin α10 promoter region were subcloned into pGL3-basic and promoter activity was analyzed in SW1353. pGL3-basic is set as one. $**p < 0.01$.

In SW1353 cells, the construct −1139 could confer high activity, the shorter constructs were also active as the construct −960 generated a 11.6-fold higher activity compared with the pGL3-basic luciferase vector, and even the −350 construct generated 9.5-fold higher activity (Fig. 3).

Identification of AP-2, Ets-1, and Sox9 binding sites on integrin α10 promoter

Sequence analysis of the integrin α10 promoter region using several data bases revealed binding sites for AP-2, Ets-1, and Sox9 in the promoter sequence (Fig. 4). The AP-2 binding sites are located between −794 and −786 (AP-2-III), −752 and −745 (AP-2-II), and −161 and −148 (AP-2-I) relative to the ATG protein start codon. The Ets-1 binding sites are situated between −474 and −469 (Ets-1-III), −172 and −167 (Ets-1-II), and −125 and −120 (Ets-1-I), two Sox9 binding sites are located between −383 and −376 (Sox9-II) and −297 and −292 (Sox9-I) relative to the ATG protein start codon.

Functional effect of AP-2, Ets-1, and Sox9 on integrin α10 promoter

To determine whether expression of AP-2 affects the transcriptional activity of the integrin α10 promoter in SW1353 cells, an expression plasmid for AP-2α was cotransfected with the constructs −350, −960, and −1139. AP-2α expression plasmid was used as it is known that AP-2α plays a crucial role in chondrogenesis, e.g., loss of AP-2α causes skeletal defects in knockout mice [12,13]. Fig. 5A shows that AP-2 increased integrin α10 expression at low doses as shown for the promoter construct −1139. Maximal activity was obtained at a dose of 10 ng of DNA/well. The integrin α10 expression was not affected by high doses of AP-2. Additionally, an antisense AP-2 construct was used, reducing AP-2 expression. SW1353 cells showed reduced integrin α10 promoter activity after downregulation of AP-2 expression. Equal results were obtained for the other two deletion promoter constructs (−350 and −960) (data not shown).

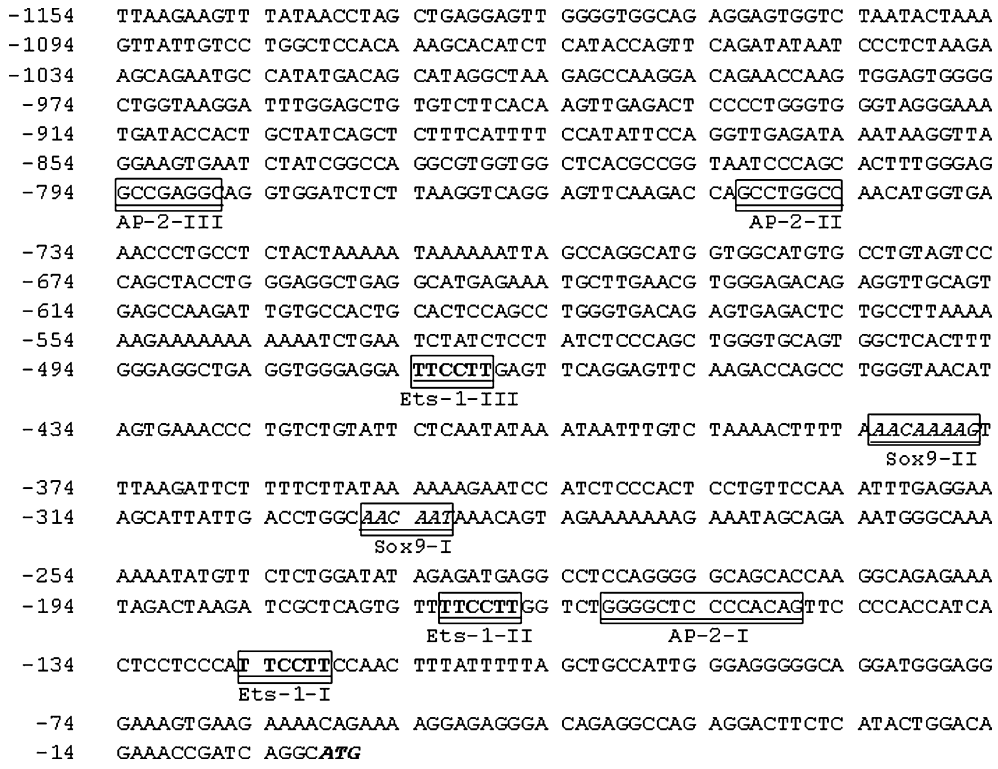

Fig. 4. Promoter sequence of integrin α10. Promoter region of integrin α10. Binding sites for AP-2, Ets-1, and Sox-9 are indicated.

Next, we studied whether Ets-1 regulates the human integrin α10 promoter in SW1353 cells. Cotransfection of an Ets-1 expression plasmid with the three promoter constructs resulted in increased integrin α10 promoter activity (Fig. 5B). Transfection of an Ets-1 expression plasmid resulted in an up to 4.7-fold induction of luciferase activity, compared to the mock transfected controls. Additionally, Ets-1 expression was down regulated using an antisense Ets-1 expression plasmid and led to a 50% decrease of promoter activity of all three constructs (Fig. 5B).

Finally, we analyzed the influence of Sox9, Sox5, and Sox6 on integrin α10 promoter activity. Interestingly, cotransfection of a Sox9, Sox5 or Sox6 expression plasmids did not alter integrin α10 promoter activity in our cells (data not shown).

Gel mobility shift assays to confirm AP-2 binding

We generated double-stranded oligomeric fragments (AP-2-I, AP-2-II, and AP-2-III) corresponding to the three regions with a possible AP-2 binding site identified with database analyses (see Fig. 4). To test whether these fragments bind to any protein factor that might regulate the integrin α10 promoter activity in the chondrosarcoma cell line SW1353, we performed gel mobility shift analyses. Several strong DNA protein complexes were observed for the fragments AP-2-I and AP-2-III using SW1353 cell nuclear extract (Fig. 6A). The fragment AP-2-II showed no shifted complexes (data not shown). In order to analyze whether AP-2 proteins indeed bind to these regulatory elements, we performed competition experiments with oligos displaying a consensus binding site for AP-2 [4] and by AP-2-I and AP-2-III, respectively. As shown in Fig. 6A (lanes 3, 4, 6, and 7), both unlabeled oligos harboring the AP-2 consensus sequence and the AP-2-I and -III oligonucleotides effectively inhibited the binding of the probe, suggesting that AP-2 or related proteins bound to the integrin α10 promoter.

To confirm the binding of AP-2, the labeled fragments AP-2-I and AP-2-III were incubated with SW1353 nuclear extract and antibodies directed against AP-2. Interestingly, addition of an anti AP-2α/β antibody did not alter the electrophoretic mobility pattern of the DNA–protein complex (Fig. 6B, lanes 5 and 10).

Therefore, we performed RT-PCR analyses to test whether other AP-2 isoforms are expressed in chondrocytes and in the chondrosarcoma cell line SW1353. We could show that, beside AP-2α and AP-2β, which are both expressed in chondrocytes and SW1353 cells, the AP-2γ gene is only expressed in SW1353 cells and AP-2δ is expressed in neither of them (data not shown). Interestingly, AP-2ε was found to be expressed in chondrocytes and in the chondrosarcoma cell line SW1353 (Fig. 6C). To examine AP-2ε expression in cartilage, tissue samples were immunostained with an antiserum against AP-2ε. Representative sections are presented in Fig. 6D. Chondrocytes were positive for AP-2ε expression and showed a nuclear staining pattern.

Having confirmed the expression of AP-2ε in chondrocytes and the chondrosarcoma cell line SW1353 we used

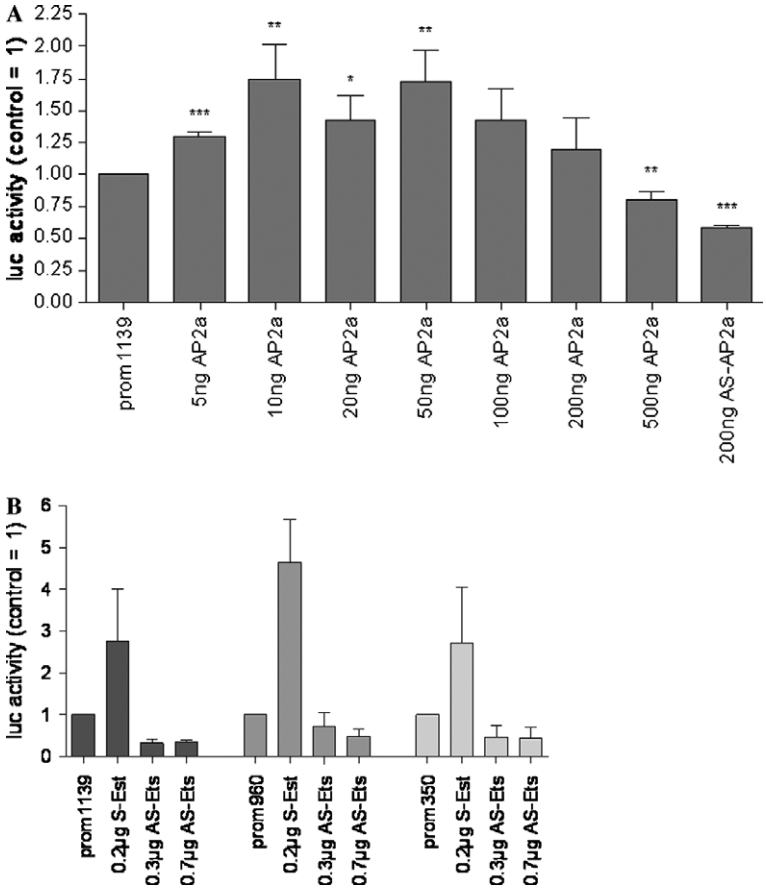

Fig. 5. Regulation of integrin α10 promoter by AP-2 and Ets-1. (A) AP-2 upregulated integrin α10 promoter activity (−1139 pGL3-basic) in a dose-dependent fashion in SW1353. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) Integrin α10 promoter constructs −350, −960, and −1139 were transiently transfected into SW1353 chondrosarcoma cells in combination with expression plasmids for Ets-1, or antisense plasmids for Ets-1, respectively. Reporter gene expression revealed promoter regulation by the transcriptional regulators.

an antiserum against AP-2ε to test whether AP-2ε specifically binds to the integrin α10 promoter (see Fig. 6B). A supershifted DNA–protein complex was obtained by incubating the labeled fragments with an anti-AP-2ε antiserum confirming the specific binding of AP-2ε to the integrin α10 promoter (Fig. 6B, lanes 4 and 9).

Discussion

In this study, we aimed to analyze regulatory factors that control integrin α10 expression in chondrocytes. Here, we present evidence for the involvement of the *trans*-acting factors, AP-2, Ets-1 but not Sox-9 in the regulation of integrin α10 transcription.

AP-2 functions are mediating the regulation of gene expression in response to a number of different signal transduction pathways. Restricted spatial and temporal expression patterns of AP-2 have been detected in several embryonic tissues, in particular in neural crest-derived cell lineages and in limb bud mesenchyme during the developmental stage when they are known to be retinoid-sensitive [23]. Our deletion analysis of the integrin α10 promoter identified a fragment (−1139) that includes three AP-2 motifs and generates a high level of expression specifically in chondrocytes. EMSAs using nuclear extract of SW1353 cells and labeled oligonucleotides displaying binding sites for AP-2 confirmed these findings for two of the three AP-2 sites (AP-2-I and AP-2-III). By EMSA using a specific antibody it could be demonstrated that the transcription factor AP-2ε binds to the integrin α10 promoter. We could also confirm that AP-2ε is expressed in chondrocytes, in SW1353 chondrosarcoma cells and in vivo in articular cartilage. Previous reports demonstrated AP-2ε expression in skin [8] and in the central nervous system [9,24]. To validate this interesting finding further analyses are necessary to investigate AP-2ε function in chondrogenesis.

AP-2α recently has been associated with the skeletal defects in mice [12,13]. The AP-2α knockout mice exhibited exencephaly, craniofacial defects, and thoraco-abdominoschisis. Since no AP-2ε knockout mouse has been described so far, it remains speculative whether AP-2ε deficient animals develop similar skeletal defects as seen in integrin α10 mouse mutants. However, due to the coexpression of various AP-2 isoforms in cartilage the loss of AP-2ε might be compensated by other AP-2 proteins.

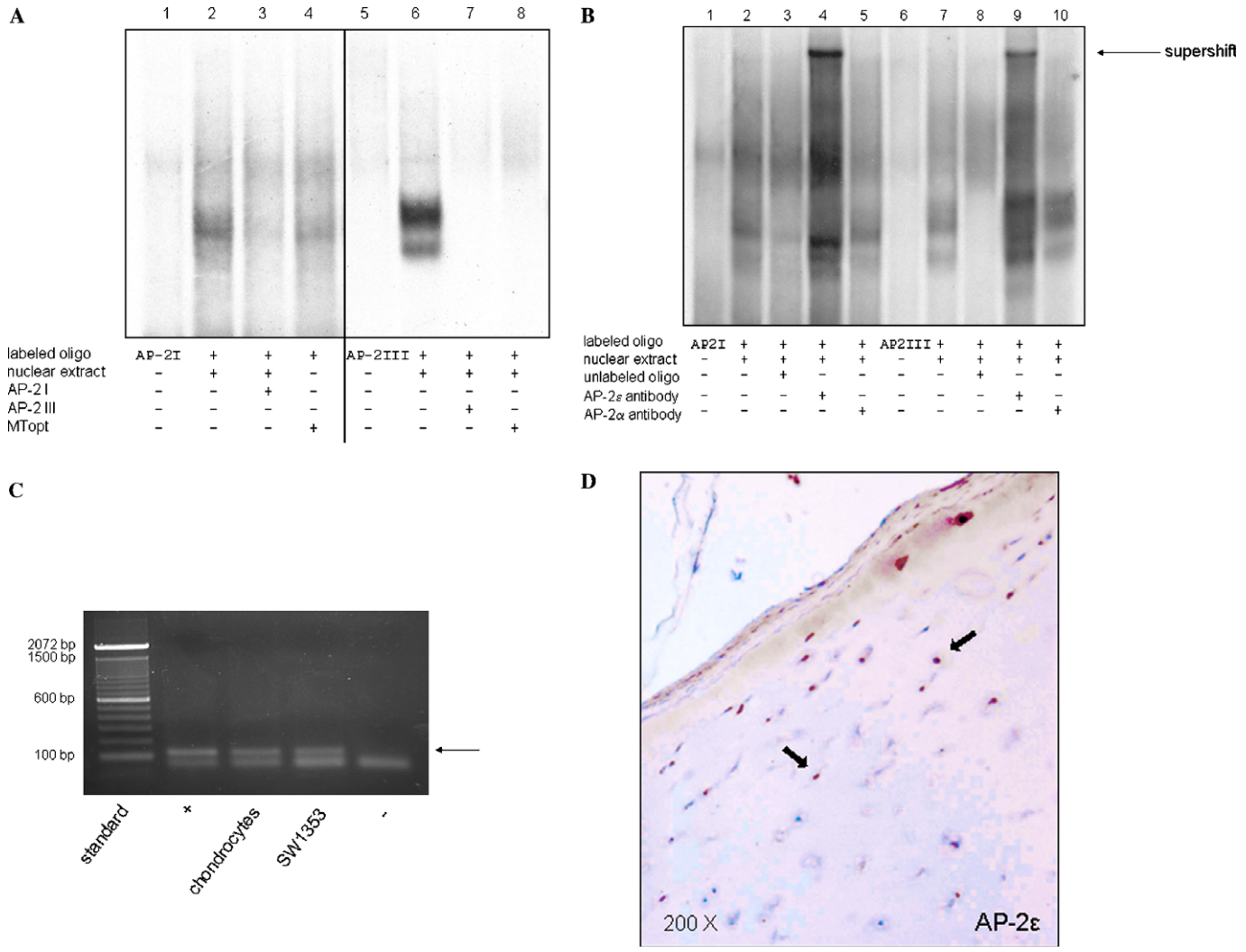

Fig. 6. Gel mobility shift assays to confirm AP-2ε binding to the integrin α10 promoter. Contents of the reaction mixtures are marked in the table below the image of the gel shift. Components which were incubated in a reaction mixture are marked with a plus. (A) The AP-2 binding was confirmed using oligonucleotides spanning the AP-2 regions I (lane 2) and III (lane 6) of the integrin α10 promoter and nuclear extract of SW1353. For competition experiments unlabeled oligonucleotides AP-2-I (lane 3), AP-2-III (lane 7), and a consensus oligonucleotide MTopt (lanes 4 and 8) were used. Lanes 1 and 5 show the labeled oligonucleotide incubated without nuclear extract. (B) Supershift assay with AP-2 antibodies. The antibodies against AP-2α and AP-2ε were incubated with the nuclear extract of SW1353 cells and the labeled oligonucleotides (AP-2-I and AP-2-III). Lanes 4 and 9 show the supershifted complex as indicated by the arrow. (C) RT-PCR to analyze the expressed AP-2ε mRNA in chondrocytes and SW1353 chondrosarcoma cells. The reaction mixture with cDNA of a positive control is marked with +, the negative control without cDNA is marked with −. (D) Immunohistochemical staining of AP-2ε revealed strong signals in cartilage. Black arrows exemplarily mark positive stained cells.

AP-2 can both activate and inhibit gene expression in genes other than integrin α10. For example, a biphasic response has been observed in the regulation of expression of insulin-like growth factor-binding protein-5 gene or MIA (CD-RAP) gene after transfection of AP-2 expression plasmid [25,26].

Our results showed a similar biphasic response for integrin α10. Exogenously added AP-2 expression vector increased transcription of the transfected integrin α10 promoter in SW1353 cells in a dose-dependent way. Altogether, these results suggest that AP-2 may play an important role in the control of integrin α10 expression.

Transcription factors other than AP-2 may also be crucial for integrin α10 expression in chondrocytes. As our experiments revealed Ets-1 acts as an enhancer for integrin α10 expression. Other factors belonging to the Ets family have been described previously to play a role in cartilage development. For example, a novel variant of Ets transcription factor chERG, called C-1-1, was cloned and characterized which is involved in regulation of cartilage development [27].

Sox9 has recently been shown to be involved in the control of the cell-specific activation of *COL2A1* in chondrocytes and to directly regulate the type II collagen gene in vivo [19,28]. However, a role as a transcriptional regulator for the expression of integrin α10 could not be confirmed for Sox9.

In summary, this is the first report on regulation of integrin α10 gene, which is specifically expressed in chondrocytes. Additionally, these results support the functional

role of AP-2ε in cartilage differentiation and show for the first time involvement of Ets-1 in regulation of gene expression in chondrocytes. The results are also significant in light of the recent reports of a severe skeletal phenotype resulting from disruption of the mouse AP-2α gene [12,13].

Acknowledgments

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