THE ROLE OF *CIS* ELEMENTS IN THE TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF THE MATRILIN-1 GENE

Ph.D. thesis

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INTRODUCTION

Matrilins constitute a novel family of oligomeric multidomain proteins, which are integrative components of various extracellular matrices (1). Matrilin-1 (previously called cartilage matrix protein, CMP) (2) is an extracellular glycoprotein synthesized by chondrocytes in a developmentally regulated manner. It is a homotrimer of 54 kDa subunits assembled via a coiled-coil α-helix and stabilized by disulfide bridges. It interacts with cartilage proteoglycans and forms both collagen dependent and independent fibrils suggesting a role in the assembly of the cartilaginous matrix. Based on recent data, matrilin-1 can be considered as a marker protein for the late proliferative stage (stage Ib) of chondrocyte differentiation. To get a deeper insight into the regulatory mechanisms responsible for the fine-tuning of cartilage-specific gene expression, we analyzed the transcriptional and post-transcriptional regulation of the chicken matrilin-1 gene. We wanted to identify and characterize of nucleoprotein complexes (enhancesomes) are involved in the efficient expression of matrilin-1 gene.

MATERIAL AND METHODS

Conventional recombinant DNA procedures were performed according to the standard protocol (5). Primary cell cultures (CEC, HDM, CEF) were made from chicken embryos (4) and transiently transfected with DNA by the calcium phosphate coprecipitation method, and assayed for CAT activity (4).

Nuclear extracts were prepared as described earlier (4). *In vitro* DNA-protein interaction studies (EMSA, *in vitro* footprinting) were done as described earlier (4). *In vivo* DNA and UV footprinting study was performed on the basis of Gerd Pfeiffers description (6). GST fusion protein expression and purification was done according to the supplayers protokol. Protein-protein interaction (GST pull-down, co-immunoprecipitation) experiments were done as described in the following article (7).

RESULTS AND DISCUSSION

The understanding of transcriptional regulation of the matrilin-1 gene is still limited. Previously, we identified the promoter and the major control regions of the

chicken gene in transient expression experiments utilizing *in vitro* culture systems of chondrogenesis.

To identify, which control regions might be involved in the regulation of cartilage-specific gene expression, we performed an functional mapping of the first intron. An overlapping deletion series of the first intron was created and its effect on the minimal promoter was monitored in transient expression studies. One of the deletion constructs showed high CAT activity compared to the other members of the series. This newly identified enhancer of the matrilin-1 gene shows similarities both location and tissue specificity to the enhancer of the type II collagen gene. While the minimal promoter showed a promiscuous activity in all cell lines tested, the mapped intronic enhancer conferred tissue specific expression at all stages of chondrogenesis.

To address the question, what kind of trans-acting factors are involved in transcriptional control of the matrilin gene, we searched for consensus binding sites in the major control regions. Putative binding sites for Sox proteins and NF-I were found in the enhancer fragment. In *in vitro* DNase I footprint analysis, we localized several protected regions using rat chondrosarcoma extracts in the intronic enhancer region. The partnership of Sox proteins with particular factors has several characteristics, and some of those can be recognized in case of the IE1 element. One of these features is the juxtaposition of the recognition sites. Putative Sox-binding sites are located at both borders and in the spacer of the NF-I recognition sequence. Neither the NF-I, nor the Sox sites match completely to the consensus, still even IE1 was capable of efficient binding of both factors. Mutational analysis indicated that the spacer motif and 3' Sox sites participated in the formation of Sox9 complexes. Binding of the two factors to their adjacent sites, however, led to a stable ternary complex formation, which was disrupted by mutation in either the NF-I or the Sox9-binding sites. Using *in vivo* footprinting, we have analyzed protein-DNA interactions at the intronic enhancer region in CEC and CEF cultures. *In vivo* footprinting studies with dimethyl-sulfate (DMS) revealed some protected regions. We detected strong protection in the IE1 element. These *in vivo* data verified our previous *in vitro* findings concerned to the intronic enhancer region. In fact, pairing Sox9 off with NF-I is so important in the transcriptional control of the matrilin-1 gene

that a single point mutation in the NF-I site dramatically reduced the protein binding to the element and the transactivation in transient transfections not only by NF-I, but also by SOX9. GST pull-down and co-immunoprecipitation experiments revealed a direct interaction between the two transcription factors in the expressing cell types, but not in non-expressing cells. It is not known, which NF-I isoforms are capable of partnering with Sox9. Four NF-I genes are known, which produce several isoforms as a result of alternative splicing (8). In the cotransfection experiments, we used NF-I, which is synthesized from the homologue of the chicken NFI-C1 gene. As Sox9 is expressed by chondrocytes and chondroprogenitor cells as well, it is possible that the expression of the respective NF-I isoform leads to activation of the matrilin-1 gene during chondrogenesis. Although it requires further studies to determine the expression pattern of the various NF-I genes during skeletal development, the almost complete absence of NF-I proteins in HDM cells at day 0 is congruent with this hypothesis. Further, characteristic changes were observed in the mobilities of the nucleoprotein complexes, when nuclear extracts prepared from HDM cultures at consecutive days were used. The nuclear proteins extracted from HDM cultures at day 4 still gave rise to ternary complexes of mobilities markedly different from those extracted from CEC cultures. It is also possible that various NF-I family members compete for the NF-I site in the enhancer of the matrilin-1 gene. As NF-I proteins have been demonstrated to bind to the silencer of this gene (4), this further emphasizes the importance of NF-I family members in the regulation of matrilin-1 gene expression. Thus, the SOX-partner cooperation model (7) may explain the sequential activation of cartilage-specific genes.

It is known that post-transcriptional (splicing) processes can be also involved in the regulation of a given gene. All members of matrilin gene family harbor an AT-AC intron at conserved positions, The efficacy of the removal of AT-AC introns *in vivo* splicing studies is far below of that the GT-AG ones. One explanation for this phenomenon is that the process of intron removal is a possible post-transcriptional regulation point. Therefor, we developed a new approach to determine the splicing efficiency of the rare intron of the matrilin-1gene *in vivo* using transient expression assays. For this purpose, we created an eukaryotic minigene, which contains the bacterial chloramphenicol acetyl-transferase (CAT) gene as a reporter gene. The AT-

AC intron was inserted into the coding region of the CAT reporter gene in a way that we could get measurable CAT activity in cell lysates only, if the intron was excised precisely from the reporter gene. The order of the GT-AG and AT-AC introns in the model construct resembles to that of the endogenous matrilin-1 gene. The other possible answer to the above-mentioned phenomenon is that the constructs tested in *in vitro* assays do not contain all the elements that are necessary for the efficient splicing. We also tested this hypothesis in our *in vivo* CAT assay system. When a specific intronic region was removed, we mapped via deletion analysis of the AT-AC intron splicing enhancer. In the absence of this intronic region, we could detect significantly lower splicing activity. Protein factors responsible for binding to *cis* elements in this region are under investigation.

CONCLUSIONS

We identified a chondrocyte-specific enhancer of the chicken matrilin-1 gene. In *in vitro* and *in vivo* footprint analysis, we localized several protected regions in the enhancer fragment. We provide evidence using competitive EMSA, super shift assays that both NF-I and Sox9 is involved in the binding to this element. We provide the first evidence for cooperative binding of heterologous transcription factors Sox9 and NF-I to juxtaposed sites of this control element, leading to high level of enhancement in COS-7 cells**.** Pull-down and co-immunoprecipitation experiments revealed a direct interaction between the two transcription factors in the expressing cell types, but not in non-expressing cells.

We developed a new eukaryotic minigene system to identify *cis* RNA motifs are involved in regulation of the U12 dependent splicing process. Using this *in vivo* approach, we identified an intronic splicing enhancer that confers high splicing efficiency for AT-AC splicing machinery.

To get a deeper insight into the biological role of enhancers in the tissue- and developmental stage specific gene regulation, we plan to continue and extend our research on the structure, function of these *cis* and *trans*-acting elements.

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