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Deletion analysis of the mouse alpha 1 (III) collagen promoter

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ABSTRACT

A chimeric gene was constructed by fusing the DNA sequences containing the 5' flanking region of the mouse a1(III) collagen gene to the coding sequence of the bacterial chloramphenicol acetyltransferase (CAT) gene. Transient transfection experiments indicated that the a1(III) promoter is active in NIH 3T3 fibroblasts and BC_3H1 smooth muscle cells. The activity of the a1(III) collagen promoter-CAT plasmid is stimulated approximately ten fold by the presence of the SV40 enhancer element. Removing sequences upstream of -200 stimulates the activity of the chimeric gene eight fold. Further deletion analysis identified sequences located between -350 and -300 that were instrumental in repressing the activity of the promoter. This 50 bp region contains a direct repeat sequence that may be involved in the regulation of the mouse a1(III) collagen gene. Truncating the a1(III) promoter to -80 further stimulated expression. We propose that the positive regulatory elements of this gene appear to be located within the first 80 bp of the promoter, whereas elements located further upstream exert a negative effect on the expression of the gene. Regulation of the a1(III) gene contrasts with that of the a2(I) collagen gene, which appears to be regulated by several positive elements located in various regions of the promoter.

INTRODUCTION

Collagens are a family of structural proteins that form the biological scaffolding of many tissues (1). Since the collagen phenotype determines to a great extent the overall tissue structure, precise modulation of collagen synthesis is crucial to the function of tissues, as well as the organism as a whole. While type I collagen is the most abundant protein in higher organisms, type III collagen is found in the fibrillar network of blood vessels, lung, and skin, complementing type I collagen in these tissues.

Expression of collagen genes is regulated during development

(2,3,4), transformation (5,6,7), and by various factors (8,9). Type I and type III collagens are regulated in a similar manner by certain agents. Fibroblasts transformed with the oncogene v-src or v-mos synthesize ten to twenty fold less type I and type III collagen mRNA (6,7). The levels of both collagens are affected by TGF-B (8), as well as hepatic fibrogenic factor (9). Since both proteins are commonly expressed by the same cells, and are affected by the same agents, it can be postulated that both genes may share common regulatory elements.

Regulatory elements that control both the temporal and tissue specific expression of genes are most often located 5' to the transcriptional start site of the coding sequence (10). Several type I collagen genes have been isolated and their promoter sequences identified (11,12,13,14). The mouse a2(I) collagen promoter has been analyzed both by deletion mutations and by small substitution mutations using a CAT recombinent vector and the transcient transfection assay in fibroblasts (15,16). The transciptional initiation site of the mouse a1(III) gene has previously been identified, and the sequences upstream of this site sequenced (17). In order to study the function of this upstream region, a 2.3 Kb fragment containing the promoter sequence was placed in front of the "reporter" gene CAT (18). The activity of the promoter was studied by introducing a recombinent a1(III) collagen promoter-CAT gene construction into cultured cells, and measuring the levels of CAT protein synthesized.

MATERIALS AND METHODS

Cell lines: NIH 3T3 and CV1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 2mM glutamine and Penicillin-Streptomycin (50U/ml, 50 ug/ml). BC_3H1 cells were maintained in DMEM supplemented with 20% fetal calf serum, 2mM glutamine and Penicillin-Streptomycin (50U/ml, 50 ug/ml). BC_3H1 cell line is derived from smooth muscle cells (19).

Plasmid constructions. Plasmid pPrC3 was constucted by replacing the a2(I) collagen promoter of pAZ1009 (15) with a 2.3 Kb a1(III) collagen promoter fragment. The vector of pAZ1009 is a

derivative of pSV0CAT, where a SV40 enhancer sequence has been inserted 3' of the CAT gene. The 2.3 Kb fragment originally an Eco R1-Acc 1 fragment, from the genomic clone lambda C35A had the Eco R1 site converted to an Xho site, and the Acc 1 site converted to a Hind III site (Figure 1). In plasmid pMM104 the SV40 enhancer sequences were deleted. All further constructions are based on pMM104. Plasmids pMM102, pMM103, and pMM110 utilized restriction sites Bgl II, Ava I, and Xba I, respectively, to construct the deletion mutations. Plasmid pMM104 was linearized with the appropriate restriction enzyme, the ends were made blunt, and the DNA was ligated. Plasmid pMM114 was constructed by converting the Nde I site at -200 to an Xho I site, and ligating the 200 bp Xho I-Hind III fragment into an Xho I-Hind III vector. Plasmid pMM120 was constructed by converting a Fok I site at -80 to and Xho I site and ligating a 96 bp (-80 to +16) fragment into the above vector. Bal 31 nuclease treatment was used to generate small deletions. Plasmid pMM104 was digested with Xba I, treated with Bal 31 nuclease, the staggered ends were converted to blunt ends using the Klenow fragment of DNA polymerase, and Xho I linkers were ligated to these blunt ends. After digest with Xho I and Hind III, fragments ranging in size from 400 to 250 bp were isolate from a polyacrylamide gel, and ligated into a pMM104 vector which had been digested with Xho I and Hind III. Deletions were analyzed by sizing the Xho I-Hind III fragments on polyacrylamide gels.

Eucaryotic Cell Transfections and CAT Assays. Plasmid DNA was transfected into cultured cells by the method of Gorman et al (18). Cells were plated at a density of $2x10^5$/100 mm dish for NIH 3T3 and BC_3H1 cells, and 10^6/100 mm dish for CV1 cells. Cells were transfected with 10-15 ug of CsCl purified plasmid DNA using the $CaPO_4$ coprecipitation technique. Four hours after the addition of DNA the cells were submitted to a 15% glycerol shock for 2 min., and then placed in media containing serum. Cells were harvested 40 hrs. after the addition of the DNA, and the extracts were assayed for CAT activity according to the method of Gorman et al (18). The modification of the Gorman protocol involved addition of acetyl CoA to 2.5 mM, and extending the

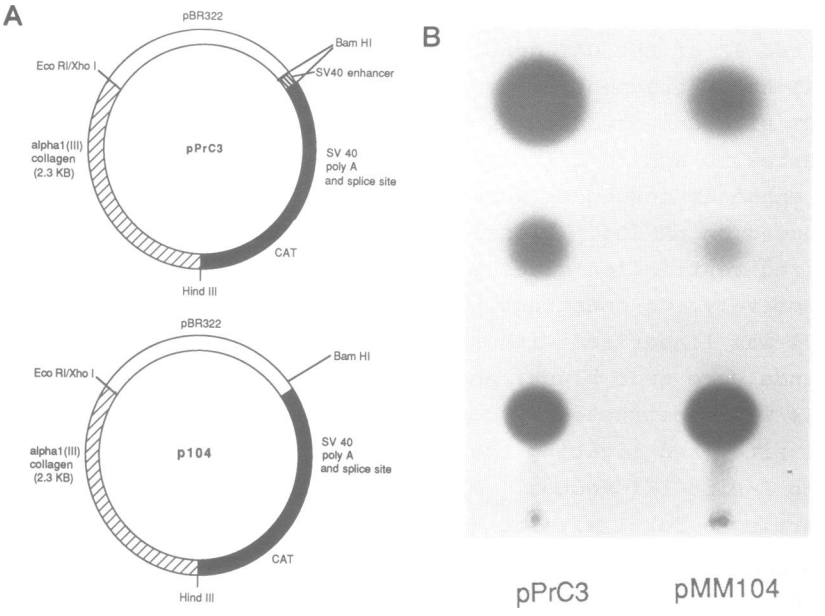


Figure 1. Schematic representation of the initial mouse a1(III) collagen promoter constructions. The stripped segments represents the 5' flanking sequence of the a1(III) collagen gene, the black region is the CAT gene, and the gray region contains SV40 mRNA processing signals. The pPrC3 construction also contains an SV40 enhancer sequence located 3' to the mRNA polyadenylation signal sequence. The right side of the panel has a representative CAT assay of the plasmids described above.

duration of the incubation to 8 -14 hrs. The data was quantitated by scintillation counting of the acetylated products.

Primer Extension Analysis. Messenger RNA transcription initiation site analysis was performed with RNA isolated from NIH 3T3 fibroblasts 40 hrs. after transfection of plasmid pMM114. Total RNA was prepared by the guanidiniun/CsCl procedure described in Maniatis et al (20). A 24-mer oligonucleotide complementary to the CAT mRNA was end labelled using [^{32}P] ATP and T4 polynucleotide kinase. Approximately 10^5 cpm (specific activity of 10^8 cpm/ug) of labelled oligonucleotide was mixed with 100 ug of total RNA from transfected or nontransfected cells in 80% formamide, 40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl and hybridized at 45^o overnight. The RNA/primer mixture was precipitated with ethanol. The nucleic acid was resuspended in 100 mM Tris-Cl pH 8.3, 10 mM $MgCl_2$, 50 mM KCl, 20 mM beta-

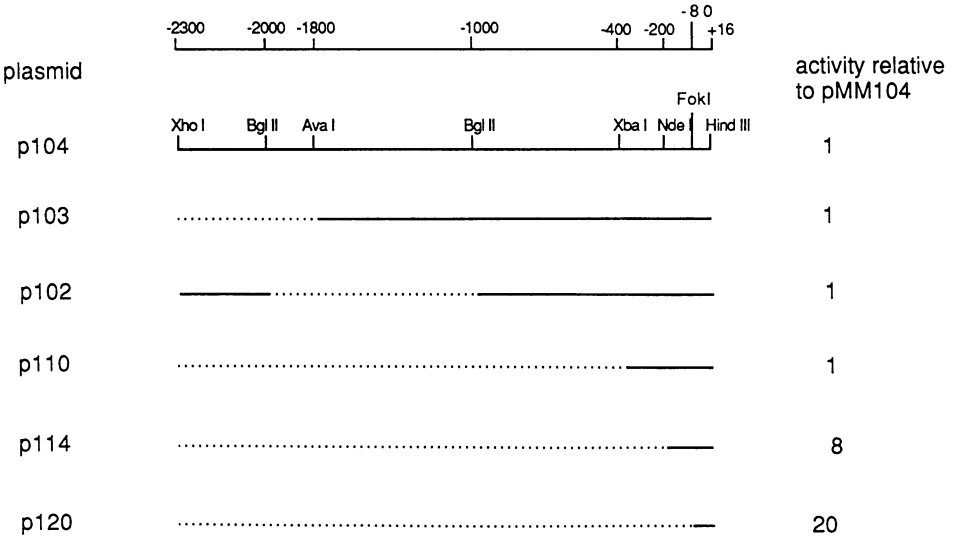


Figure 2. Schematic representation of the initial deletion construction and their activity in NIH 3T3 fibroblasts. The solid line represents DNA sequences remaining in the plasmid construction. The dotted line represents sequences that were deleted. CAT activities are expressed relative to the CAT activities obtained with pMM104.

mercaptoethanol, 2.5 mM each dNTP, and 10 units of reverse transcriptase. The reaction was incubated for 1 hr. at 37°. After phenol extraction, ethanol precipitation, and a 30 min. incubation in 0.1 N NaOH at 37°, the sample was adjusted to 5 M urea and 0.01% bromphenol blue, and boiled for 2 min. The extension products were analyzed on an 8% sequencing gel.

RESULTS

Activity of the mouse a1(III) collagen promoter. The isolation and identification of the mouse a1(III) collagen promoter has been previously reported (16). A unique transcriptional start site was contained within a 2.3 Kb 5' fragment flanking the a1(III) collagen structural gene. This 2.3 Kb fragment was cloned 5' to the bacterial chloramphenicol acetyltransferase (CAT) gene, placing the CAT gene under the control of the a1(III) collagen promoter (Figure 1). Plasmid constructions pPrC3 and pMM104 differed in that pPrC3 contained a SV40 enhancer sequence inserted 3' of the CAT gene, and pMM104 did not. Transcient transfection assays indicated that both pPrC3 and pMM 104

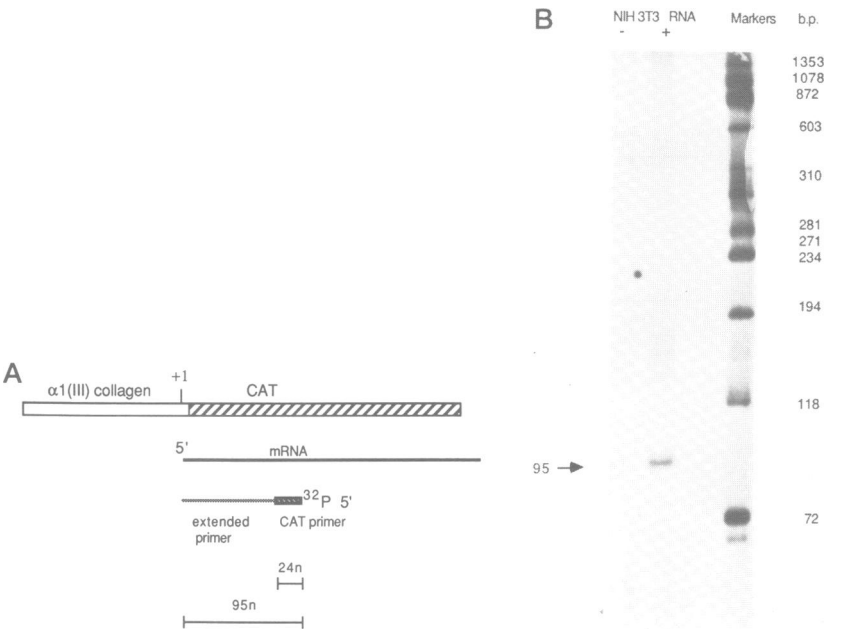


Figure 3. Primer extension analysis of mRNA isolated from NIH 3T3 cells transfected with plasmid pMM114. Panel A outlines the primer extension experiment. An autoradiogram of the primer extension products is presented in the B panel. Markers correspond to OX 174 DNA digested by Hae III and were used to estimate the size of the transcript. The 95n extended product is indicated by an arrow. + indicates RNA from NIH 3T3 cells transfected with plasmid pMM114, and - indicates RNA isolated from nontransfected NIH 3T3 cells.

constructions were active in NIH 3T3 fibroblasts, but that pPrC3 had approximately a ten fold higher activity than pMM104 (Figure 1). The activity of the mouse a1(III) collagen promoter is approximately five to ten fold lower than the activity of an analogous mouse a2(I) collagen promoter (data not shown). These levels of activity reflect the relative ratio of type III to type I collagen synthesized by cultured fibroblasts.

Deletion Analysis. The initial deletion study of the a1(III) collagen promoter involved removing large regions of the promoter. This first series of mutations removed progressively more 5' sequence, leaving the cap-proximal 3' sequences intact (Figure 2). The deletions ranged in size from 500 bp in pMM103 to over 2.2 Kb in plasmid pMM120. Deletions of sequences 5' of -400 had no affect on the level of expression. Deletion of

sequences to -200 however, increased the level of expression approximately eight fold. Further deletion to -80 resulted in still a further increase to an approximately twenty fold higher level of expression then the parent pMM104 construction (Figure 2). These deletion mutations were also transfected into CV1 cells and BH_3C1 smooth muscle cells. The deletions had the same relative activities in these cell lines as in the NIH 3T3 fibroblasts (data not shown). These results suggest that deletion of sequences between -400 and -200 remove sequences that negatively regulate expression of this gene.

Determination of the transcriptional start site. Deletion of promoter sequences to -200 also places pBR322 vector sequences closer to the CAT gene. Moving vector sequences closer to the CAT gene could result in read through transcription originating from the upstream vector region. To rule out this possibility, RNA was isolated from NIH 3T3 fibroblasts transfected with the pMM114 plasmid. The control RNA was isolated from non-transfected NIH 3T3 fibroblasts. The mRNA was hybridized to a 5' end labelled oligonucleotide complementary to CAT mRNA. The compliment of the oligonucleotide sequence is located 92 bp downstream of the transcriptional start site in pMM114. After hybridization and extension of the oligonucleotide with reverse transcriptase, the extension products were analyzed on a denaturing polyacrylamide gel. The size of the extended product was approximately 95 nucleotides, in agreement with the predicted size of 92 bp (Figure 3). There were no larger transcripts, and no extended products were found in the control reaction. This result indicated that the increase in acivity was not due to transcription originating in the adjacent vector sequences, since transcription initiated from the start site of the a1(III) collagen promoter.

The greatest increase in activity occured when sequences from -400 to -200 were deleted. To further define which sequence within this 200 bp region was responsible for this effect, Bal 31 nuclease deletions between -400 and -200 were generated and analyzed. As in the previous constructions, the 3' end of the promoter remained unchanged, only 5' end sequences were deleted. Plasmids pMM204 and pMM205 which had deleted sequences to -365

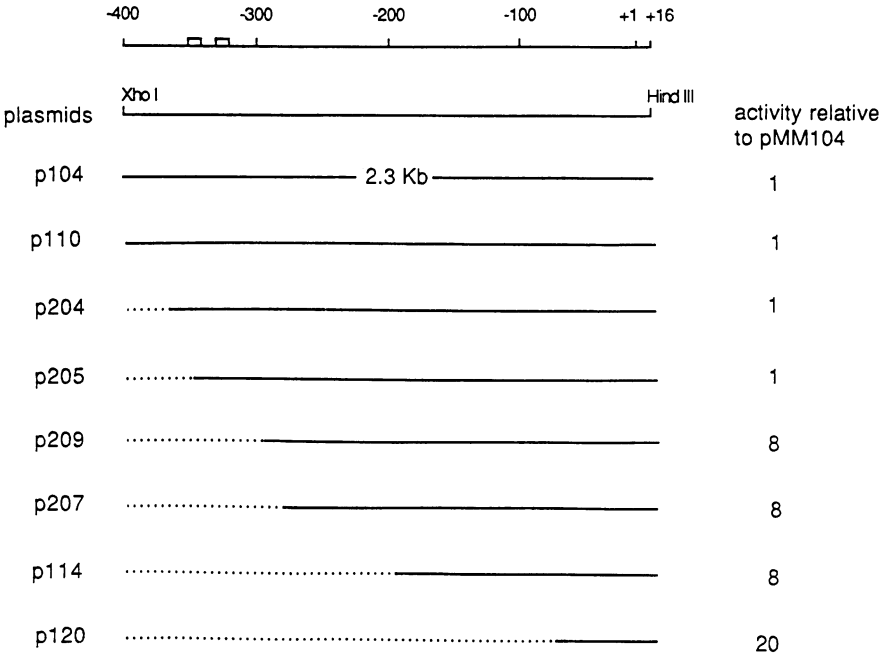
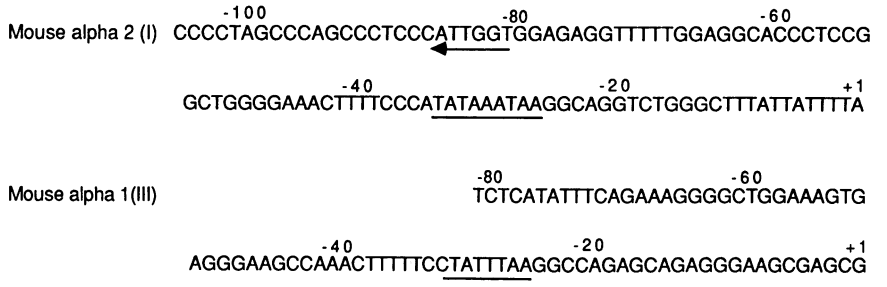


Figure 4. Mutations denerated by Bal 31 nuclease and this corresponding CAT activity in NIH 3T3 cells. CAT activities are expressed relative to CAT activities obtained with pMM104. The boxes between -400 and -300 indicate the location of the imperfect repeat sequences found in this promoter.

and -350, respectively, had the same activity as pMM110, a mutation with a deletion endpoint at -400. Plasmids pMM209 and pMM207 which had sequences deleted to -300 and -285, respectively, had the same activity as pMM114, or eight fold more activity then the parent plasmid (figure 4). These deletions were also tested in CV1 and BH_3C1 cells where they had the same relative activity as in the NIH 3T3 cells (data not shown). The progressively larger deletions did not exhibit a gradual increase in activity, but rather there was an increase when a 50 bp sequence was deleted.

The deletion mutation pMM120, which contained 80 bp of promoter sequence had a relatively high level of activity - 2.5 fold higher then the 200 bp promoter plasmid pMM114. The activity of this deletion plasmid was compared to a mouse collagen a2(I) deletion construction. Both constructions have



Plasmid	Activity relative to pMM120
pMM120	100%
p1045A1	10 - 12%

Figure 5. Comparison of the a1(III) and the a2(I) mouse collagen promoter sequences spanning the first 80 and 104 bp respectively. The start site of transcription is marked by +1, the TATA box sequence is underlined, and the CCAAT sequence (present on the template strand) is underlined with an arrow in the a2(I) sequence. The relative activity of CAT plasmid construction containing the truncated collagen promoters is shown below the sequence.

the same vector sequences. The a2(I) promoter deletion p1045A1 contains 100 bp of the mouse a2(I) collagen promoter (Figure 5). Both promoters have a TATA box, but share no other elements. The a2(I) collagen promoter deletion p1045A1 has 8 fold less activity then the a1(III) collagen deletion pMM120. This finding indicates that a) the first 80 bp of the a1(III) collagen promoter are sufficient to support transcription of the CAT gene in this recombinant vector, and b) an analogous 100 bp plasmid construction containing a2(I) promoter sequences is not sufficient for high levels of CAT expression.

DISCUSSION

The current study examines the elements regulating the mouse a1(III) collagen promoter. Initial analysis indicated that this promoter is relatively weak, having 10 to 20 percent of the activity of the mouse a2(I) collagen promoter. Subsequent deletion analysis suggested that the a1(III) promoter, unlike the a2(I) collagen, is regulated predominately by negative regulatory

elements. Further deletion studies identified a 50 bp sequences that inhibited the expression of the promoter. Truncating the a1(III) promoter further to -80 resulted in a still higher level of expression. This finding indicated that the first 80 bp of the a1(III) promoter are sufficient for a relatively high level of expression. In contrast, truncating the a2(I) promoter to 100 bp resulted in decreased expression, due to removal of positive regulatory elements located further upstream. Thus dissimilar promoters govern the expression of these two collagen genes that are often coordinately regulated in various tissues.

Two types of plasmid constructions containing the mouse a1(III) collagen 5' flanking sequences were used in the initial experiments. Including the SV40 enhancer sequences in the a1(III) CAT plasmid construction stimulated the activity of the a1(III) promoter, similar to the affect previously seen by the addition of the SV40 enhancer sequences to an a2(I) promoter (15), as well as other promoters (23,24,25). However, since enhancer elements may interfere with normal regulation, in the current study, all the deletion analysis of the a1(III) promoter were conducted with plasmid constructions that did not contain enhancer sequences.

The first series of deletions involved the removal of 5' sequences, and progressively reducing the size of the promoter. These experiments demonstrated that removal of sequences upstream of -400 did not affect the expression of the CAT gene. Further deletion to -200 increased the activity of the promoter 8 fold. Primer extension analyses of the 200 bp promoter plasmid indicated that mRNA transcription originated from within the a1(III) promoter sequence, providing further evidence that the increase in activity was dependent on the removal sequences that inhibited promoter activity.

The sequences responsible for this increase in activity were localized by Bal 31 deletion analysis. Incremental deletions indicated that sequences located between -350 and -300 were inhibiting the expression of the promoter. Inspection of this 50 bp sequence revealed that this region contains imperfect direct repeat sequences, separated by ten bp. This arrangement would place the two direct repeat sequences on the same face of the DNA

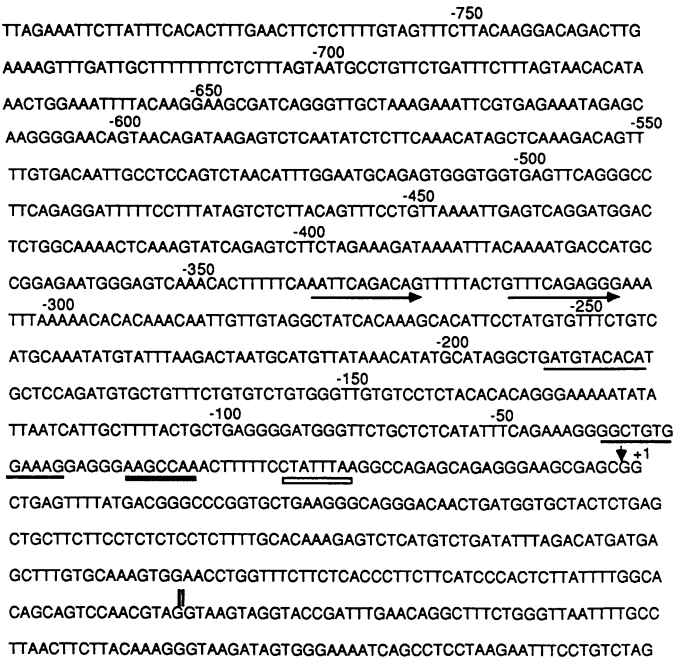


Figure 6. Nucleotide sequence of the first exon, part of the first intron, and promoter sequences. The open bar underscores the TATA box sequence, the stippeled bar - a Nuclear factor I binding site consensus sequence, thick black bar - an enhancer core consensus sequence, the horizontal arrow - sequences homologies between the a1(III) and a2(I) collagen promoters. A vertical arrow indicates the transcriptional start site, and the vertical bar indicate a potential splice site marking the end of the first exon.

helix. If this region contains a protein binding site, correct sequence alignment may by important for the protein interactions. Alternatively direct repeat sequences would allow the formation of slip structures which have been implicated in gene regulation (26). This 50 bp region contains a sequences that has partial homology with the a2(I) collagen promoter. In preliminary DNA footprint experiments, the direct repeat sequences are protected, providing evidence that this 50 bp region is involved in protein interactions (unpublished results A. Hamatochi).

Our results indicated that the 120 bp sequence located between -200 and -80 exerts a small negative affect on the expression of the a1(III) collagen promoter. In contrast, a deletion of a2(I) promoter sequences to -104 produces a plasmid that has less then one tenth of the activity of the parent 2 kb

a2(I) promoter construction. Thus deletion of upstream sequences from the a2(I) promoter removes positive regulatory elements essential for optimal activity, while deletion of comparable sequences of the a1(III) promoter removes negative elements.

Comparison of the first 80 and 104 bp of the two collagen promoters reveals that they share few elements (figure 6). The only region that is highly conserved surrounds the TATA box sequence. The a2(I) promoter has a CCAAT box sequence located at -80 (on the template strand), which binds a heterodimer that has been purified to homogeneity (20). Binding of this protein to the -80 region of the a2(I) promoter is not competed by an -80 bp region of the a1(III) promoter (21). The segment containing this sequence in the a2(I) promoter is the binding site for a trans-acting factor, which stimulates the a2(I) collagen promoter in a cell-free system (S. Meity, P. Golumbek, G. Karsenty and B. de Crombrugghe, unpublished results). Furthermore, point mutations in the CCAAT motif which inhibit binding of the factor, also show a decreased promoter activity in DNA transfection experiments (16). The absence of this sequence in the a1(III) promoter may be critical in differential regulation of these collagen genes.

The a1(III) promoter does have two sequences that may be important for the expression. A nuclear factor 1 binding site consensus sequence is located around -40 (figure 7), and a SV40 enhancer core consensus sequence is found around -52. These sequences are not present in the first 104 bp of the a2(I) promoter, and may be responsible for the high level of activity of the a1(III) truncated promoter.

Results of the deletion analysis of the a2(I) (15) and a1(III) collagen promoter suggest that the two promoters are regulated by different mechanisms. These observations must take into account that the studies were conducted primarily with cultured fibroblasts. Other tissues which express collagens at higher levels may utilize sequences that could not be analyzed in this study. However, results obtained with cultured smooth muscle cells were identical to those found with cultured fibroblasts.

Finally, regulation of transcription is only one mechanism

that regulates the expression of genes. Previous reports presented evidence that both the a1(III) and the a2(I) collagen genes may subject to translation control (27). This mode of regulation could account for one level of coordinate gene control. It is also possible that other regulatory elements are located either further upstream or perhaps downstream of the sequences analyzed in this study. This study demonstrates that promoters of two genes that are often coordinately expressed are regulated in a different manner, indicating that various regulatory mechanisms can be employed to modulate gene expression.

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