# Cooperative and competitive interactions of regulatory elements are involved in the control of divergent transcription of human Col4A1 and Col4A2 genes

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**Abstract The genes COL4A1 and COL4A2, coding for the two subunit chains α1(IV) and α2(IV) of collagen IV [α1(IV)$_2$α2(IV)] are found closely linked on the human chromosome 13 in a unique head-to-head arrangement resulting in opposite strand transcription starting from a shared promoter region. Transient transfection experiments defined a shared promoter and two symmetrically arranged, downstream located and gene-specific activating elements in each gene. The shared promoter does not exhibit any transcriptional activity and efficient transcription depends on the cooperative effect of downstream elements. Mutual inhibitory effects between the two activating elements indicate competitive interactions with the shared promoter. Symmetry, cooperativity and competitivity of cis-elements are also reflected by the binding of transacting factors to the promoter and activating elements. From these data we propose a model for the coordination of divergent transcription of COL4 genes based on the cooperative and competitive interactions of the shared promoter and gene-specific regulating elements.**



*Key words:* Collagen IV; Regulation; Promoter; COL4A1; COL4A2

## 1. Introduction

Collagen type IV is the major structural element of all basement membranes found in highly developed organisms [1,2]. The heterotrimeric molecules with the subunit structure α1(IV)$_2$α2(IV) form a complex multilayer network [3–5] that determines the macromolecular organization and the biomechanical stability of the basement membrane zone [1]. Specific interactions of collagen type IV with other basement membrane constituents regulate their incorporation into the network and are also responsible for the ordered adherence of cells to basement membranes. Besides the α1(IV) and α2(IV) chains present in all basement membranes, four additional isoforms, α3(IV)–α6(IV), have been identified with distinct expression patterns [6].

The human genes COL4A1 and COL4A2 encoding the subunit chains α1(IV) and α2(IV), located close to each other on chromosome 13q34 [7], were found to be orientated head-to-head and are therefore transcribed in divergent directions [8,9]. An identical genomic arrangement was detected for the corresponding murine genes [10,11]. A similar organization was recently seen also with the COL4A3-COL4A4 [12] and COL4A5-COL4A6 gene pairs [13]. Different from mammalian genomes, the COL4 genes in *Caenorhabditis elegans* are located on different chromosomes [14]. The transcriptional unit of the collagen type IV genes is unique, since it coordinates the cell-specific production of two subunits essential for the formation of the final protein. Other bidirectional promoter systems, as found in viral [15,16], yeast [17–19] or mammalian genomes [20–22] differ in the distances of initiation sites, their ability for coordinated expression and the fact that linked genes do not represent the subunits of a multimeric protein.

Previous studies revealed that the promoter itself is not transcriptionally active [8] and efficient transcription depends on the presence of downstream located elements within both genes [23]. A strong enhancing element was detected in the murine COL4A1 gene [10,24], but no corresponding element could be detected in the human homologues (R. Pollner, unpublished). Otherwise, we identified a strong negatively acting element, the COL4 silencer, within the third intron of the COL4A2 gene [25]. At least three nuclear factors, a CAAT-binding protein, Sp1 and a novel factor, named CTCBF, are able to interact specifically with the promoter as well as downstream located elements [23,26]. Mutational studies substantiated the involvement of the promoter-bound factors in transcription initiation and defined the shared promoter to represent an overlapping region of two gene-specific promoters using the same elements with differential efficiency [23].

In this paper we present an analysis of the interplay of essential elements in the expression of the COL4A1-COL4A2 genes and present a model based on the cooperative and competitive interactions of gene-specific activating elements with their shared promoter.

## 2. Materials and methods

### 2.1. Construction of plasmids

All constructs used in transfections are based on the plasmid vector pBLCAT2 [27]. Different genomic fragments (numbering according to EMBL: HSCOL4A12) were included (compare Figs. 1 and 2) and the generated chimeric constructs were characterized by restriction mapping and partial sequencing. An asterisk indicates sites made blunt before ligation, sites in brackets refer to vector sites and numbers in brackets refer to nucleotide positions. **pNB**: 0.35 kb *Bam*HI-*Nco*I* (6019–6365), made blunt with mung bean nuclease to delete the α1(IV)-specific ATG codon, in pBLCAT2/*Bam*HI*-*Bgl*II; **pBN**: same fragment in *Bam*HI-*Bgl*II* of p0CAT; **pNA** and **pAN**: 0.87 kb *Nco*I*-*Apa*I* (6019–6866) subcloned into *Bam*HI*-*Bgl*II* digested pBLCAT2 in both orientations; pNB/BA and pNB/AB: 0.5 kb *Bam*HI*-*Apa*I* (6365–6866) inserted into *Kpn*I* of pNB in both direc-

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*Abbreviations:* COL4, collagen IV; CAT, chloramphenicol acetyl transferase; hGH, human growth hormone


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tions; **pEN**: the 1.5 kb *Sph*I-*Eco*RI-(*Hin*dIII) fragment (6739–7886) from p361 [8] was added to *Sph*I-(*Hin*dIII) digested pAN. **pBNaSA**: was constructed in multiple steps. First, the 0.72 kb *Sph*I-*Ncol**-(*Sph*I) fragment of pNA was deleted, generating pSA (not shown), containing the 0.13 kb *Sph*I-*Apa*I* (6739–6866) fragment linked to the CAT cassette. Then, the 1.8 kb (*Hin*dIII-*Kpn*I) fragment of pSA was inserted into pUCBH, which contains the 6.4 kb *Hin*dIII-*Bam*HI (1–6366) fragment in the vector pUC931. From the resulting pBHSA (not shown), the 5.6 kb *Hin*dIII-*Nae*I (1–5644) sequence was deleted in two steps: removal of the 4.5 kb *Hin*dIII*-*Xba*I* fragment (1–4559), pBXSA (not shown), and the removal of a 1.1 kb *Xba*I*-*Nae*I fragment (4559–5644). The α1(IV)-specific ATG (6015–6017) was deleted by cleavage with *Nco*I (6014) and treatment with mung bean nuclease. **pHA**: 6.4 kb *Hin*dIII-*Bam*HI (1–6366) was inserted into *Bam*HI-*Hin*dIII cut pNA. **pXA**: deletion of 4.6 kb (*Xba*I)-*Hin*dIII-*Xba*I fragment (1–4559) from pHA; **pXENA**: insertion of the 1.15 *Xba*I*-*Eco*RI* (4559–5704) fragment into the *Xba*I* site of pNA and determination of orientation by restriction mapping; **pANaSA**: insertion of the 0.6 kb *Pst*I-(*Pst*I) of pAN in *Pst*I cut BNaSA; **pBNBA**: inversion of the 0.35 kb (*Xba*I*)-*Nco*I*-*Bam*HI* (6019–6366) fragment of pNA. **pNBNNaSA**: the intermediate BXSA was cut with *Nco*I and (*Sal*I), treated with mung bean nuclease, recircularized (pNXSA; not shown) and the 0.35 kb (*Xba*I*)-*Nco*I*-*Bam*HI* fragment (6019–6366) of pNA was inserted in *Pst*I* cut pNXSA. Finally, the 1.1 kb *Xba*I*-*Nae*I fragment (4559–5644) was deleted. For use in transient transfections, supercoiled plasmid DNA was isolated by two successive CsCl gradients.

*2.2. Cell culture and transfections*

HT1080 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For transfection, $2\times10^5$ cells were plated 16 h prior to the addition of DNA. Supercoiled plasmid DNA was transfected by calcium co-precipitation or in later experiments by liposome fusion. To eliminate variations in transfection efficiency, CAT constructs were co-transfected with pL51, containing the β-galactosidase gene driven by the LTR of the RSV virus (gift from R. Nischt, Cologne). Cells were harvested 48 h after transfection and whole-cell extracts were analyzed for CAT and β-galactosidase activity as described [8]. Values represent the average of three independent transfections, each done in duplicate assays. Activities were calculated relative to the activity of pBLCAT2 arbitrarily set at 100%. The promoterless construct p0CAT detected non-specific transcription rates.

*2.3. Nuclear runoff transcription*

Nuclei of $2\times10^7$ HT1080 cells were isolated and nuclear run-off transcription reactions were done essentially as described [28]. 3–5 mg of isolated cDNA inserts or M13 phage DNA was denatured, blotted onto nitrocellulose filters and hybridized to constant amounts of labeled RNA at 65°C for 48–72 h. The filters were washed in 2×SSC at 65°C for 1 h, treated with RNAase and exposed to preflashed X-ray film. The following probes were used: α1(IV) cDNA fragment, 3.8 kb (pos. 1543–5348); α2(IV) cDNA fragment, 4.3 kb (pos. 353–4635) [29]; $tRNA^{Val}$, 0.8 kb genomic fragment [30]; pUC19; antisense CAT, 1.6 kb *Bgl*II-*Sma*I fragment of pBLCAT2 subcloned in M13tg130; antisense hGH, 2.2 kb *Xba*I-*Eco*RI fragment of p0GH [31] subcloned in M13tg130; 0.45 kb β-actin cDNA fragment and 0.3 kb β-tubulin cDNA fragment. Relative rates of transcription were calculated after densitometric analysis of autoradiographs and direct measurement of bound radioactivity.

Nuclear run-off transcription analysis of transfected cells was done similarly. About $2\times10^5$ HT1080 cells were plated and transfected by lipofection with either 15 mg pNA or pNB, together with 10 mg pTK-hGH. Nuclei were harvested 48 h after transfection and $5\times10^6$ nuclei were used for transcription assays.

## 3. Results

### *3.1. Activating elements are located within the COL4A1 and COL4A2 genes*

The genomic region surrounding the shared promoter of the human COL4A1-COL4A2 genes was characterized as described earlier [8] and the arrangement of exons and the orientation of transcription initiation sites is shown in Fig. 1A. To search for basic regulatory elements we generated sets of chimeric constructs containing the genomic fragments linked to the CAT reporter gene (Fig. 1B) and tested for their activity after transfection to the human fibrosarcoma cell line HT1080 [32]. Similar results were obtained with alternative collagen IV synthesizing cells, such as A431, HBL100 or Tera2 (data not shown). Constructs detecting the transcription of the COL4A2 gene (Fig. 1B), which contained either the promoter alone (pNB) or additional upstream located regions (pXB, pEB), did not result in significant levels of activity. Similarly, no activity was seen with constructs pBN, pAN and pEN, detecting transcription of the COL4A1 gene (Fig. 1C). Therefore, the promoter exhibits no intrinsic transcriptional activity in either direction and addition of upstream located regions did not stimulate transcription in any direction.

A strong increase of the COL4A2 specific transcription, however, was detected after inclusion of the first intron (330 bp) of the COL4A2 gene, as seen with pNA (Fig. 1B). Therefore, the additional fragment BA (see Fig. 1A) located downstream of the transcription initiation site, contains a positively acting element. In contrast to typical enhancers [33], its activity strongly depends on the correct genomic arrangement. When this sequence was placed downstream of the CAT cassette, as in the constructs pNB/BA and pNB/AB (Fig. 1B), its activating effect was lost. The lack of any activating effect of this region on transcription of the COL4A1 gene, as seen with pAN and pEN, indicates that the fragment BA contains a gene-specific, unidirectionally activating element. The palindromic organization of the COL4A1-COL4A2 locus suggested the idea that a functionally corresponding element may also be located within the COL4A1 gene. This was tested by the inclusion of 289 bp of the first intron of the COL4A1 gene in pBNaSA (Fig. 1C). The start site of translation in the first exon of the COL4A1 gene was mutated to enable proper expression of the CAT reporter gene and fragment SA (Fig. 1A) was included as a splice acceptor site. The final construct pBNaSA exhibited enhanced transcription when compared with the inactive promoter constructs pBN, pAN and pEN. These experiments confirmed that expression of the COL4A1 gene also depends on a gene-specific activating element located downstream within the first exon and intron.

The functional dependence of both activators on their shared promoter implies mutual competitive effects between both elements. This notion was tested by including both activators in the same construct. The activity of pXA, detecting transcription in α2(IV) direction, decreased to about 50%, when compared with the construct pNA (Fig. 1D). However, full activity was restored by deleting the fragment EN in pXENA, which resembles the COL4A1 activating element. Similar results were obtained with constructs detecting transcription in direction of the COL4A1 gene (Fig. 1E). Addition of the α2(IV) activating element in the construct pANaSA resulted in a decrease of transcriptional activity when compared with pBNaSA.

The symmetrical arrangement of the gene-specific activating elements and the partial palindromic sequence of the promoter [8] suggested that the promoter may be functionally bidirectional when combined with activating elements. This was tested by the constructs pBNBA and pNBNNaSA (Fig. 2) containing the fragment NB (Fig. 1A) in reversed orienta-

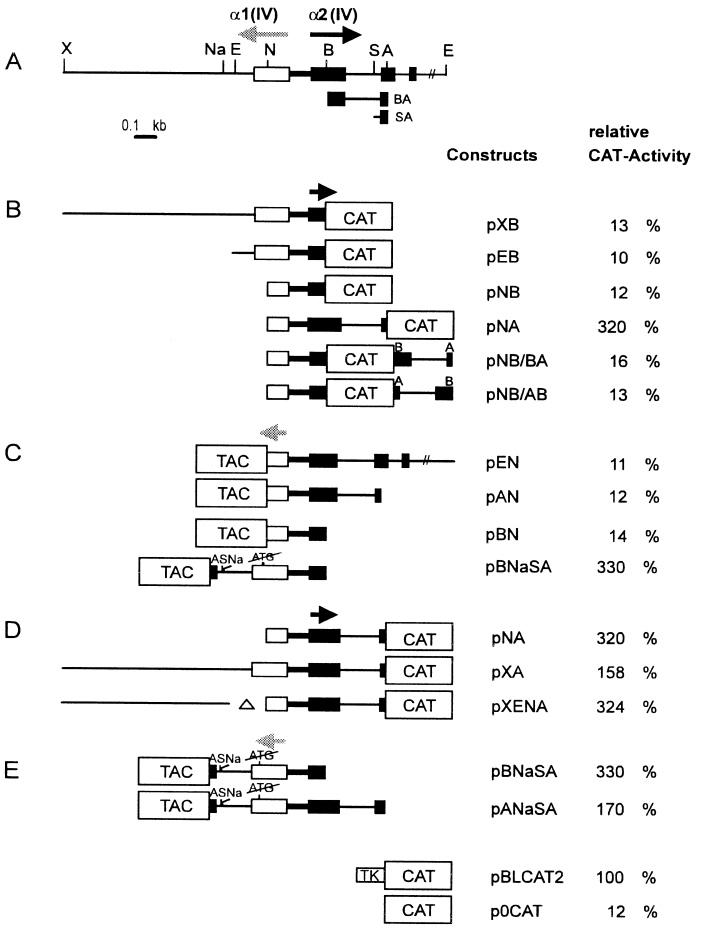


Fig. 1. Identification of activating regions within the COL4A1 and COL4A2 genes. A: Genomic map of the human collagen type IV promoter region. Exons of the COL4A1 (open boxes) and the COL4A2 gene (black boxes), introns (thin lines), shared promoter (thick line), the orientation of bidirectional transcription [8] and the fragments BA and SA used during construction of plasmids are indicated. Restriction sites are indicated by N: *Nco*I, B: *Bam*HI, X: *Xba*I, Na: *Nae*I, S: *Sph*I and E: *Eco*RI. B–E: Transient transfection assays of chimeras in HT1080 cells. The relative transcription activities were normalized for the activity of pBLCAT2, set arbitrarily as 100%. The level of non-specific transcription is represented by p0CAT.

tions. No gross changes in organization and relative distances of elements were introduced. Transcriptional activities were found to be comparable to the corresponding constructs pNA and pBNaSA and indicate that the promoter is functional in both orientations when combined with downstream gene-specific activating regions of any gene.

### 3.2. *The COL4A2 activating element stimulates transcription initiation*

Stimulatory effects of additional introns with the CAT cassette on post-transcriptional events have been observed in some systems, which may corroborate transfection results [34]. In order to confirm that observed CAT activities reflect transcriptional activity, we determined mRNA synthesis by nuclear run-off transcription assays of HT1080 cells transfected with the constructs pNA and pNB (see Fig. 1), which include or lack the first intron of the COL4A2 gene, respectively (Fig. 3A). Co-transfection with pTK-hGH [31] was used to eliminate potential variations in transfection efficiencies. Specific labelled nuclear transcripts from the plasmid templates were assayed by hybridization to membrane-bound antisense probes represented by single-stranded DNA from M13 clones corresponding to the coding sequences of the CAT or hGH gene. The relative ratio of CAT- and hGH-

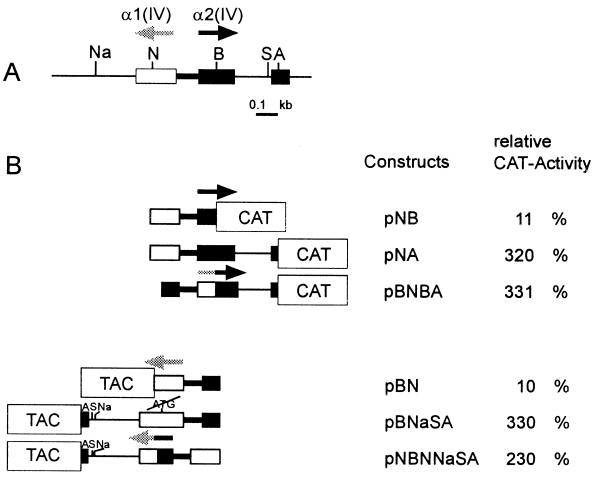


Fig. 2. The shared promoter is transcriptionally inactive, but functional in either orientation with both activators. A: Genomic map as described in Fig. 1. B: Constructs containing the promoter (fragment NB: pos. 6019–6365) in reversed orientation, pNBNNaSA and pBNBA, show comparable transcriptional activities when compared to constructs pNA and pBNaSA, reflecting the authentic orientation of the promoter (see Fig. 1).

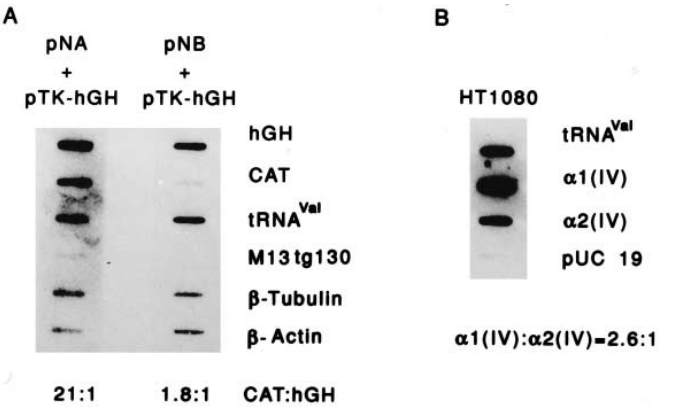


Fig. 3. Determination of rates of transcription initiation in vivo. A: Nuclear run-off transcription assays of HT1080 cells were performed after co-transfection with either pNA or pNB (see Fig. 1) together with pTK-hGH. Labeled mRNA transcripts were hybridized to filter-bound antisense probes for the coding sequences of the CAT and hGH genes. A genomic fragment of $tRNA^{Val}$ and cDNA fragments of β-tubulin and β-actin were used as positive and M13tg130 phage DNA as negative controls. B: Determination of the relative rates of bidirectional transcription in vivo by nuclear run-off transcription of HT1080 cells. Specific transcripts were detected by antisense cDNA probes for α1(IV) and α2(IV) with the indicated length. A genomic fragment of the $tRNA^{Val}$ gene was used as positive and pUC19 as negative control. Filter-bound radioactivity was determined and relative rates of transciption were calculated after normalization for length of the probes.

specific transcripts was used as an indication of the level of transcription. Cells transfected with pNA exhibited a 12-fold increased relative rate of transcription when compared to cells transfected with pNB. Constant levels of expression were detected for the β-actin and β-tubulin genes as positive control. No background signals were seen with M13 phage DNA. Therefore, the stimulatory effect of the first intron is mainly due to a stimulatory effect on transcriptional activation.

The qualitative and quantitative results of the transfection experiments suggested a ratio of 1:1 for bidirectional transcription of COL4A1 and COL4A2 (see Fig. 1B,C). Therefore, the relative rate of divergent transcription in vivo was measured by nuclear run-off transcription using HT1080 cells (Fig. 3B). A ratio of 2.6:1 was observed for the relative synthesis of the α1(IV) and α2(IV) mRNAs. A preferred transcription of the COL4A1 gene was also detected for some other cell lines [35], a result consistent with earlier reports, that described an excess of α1(IV) mRNA in different cells [36].

## 4. Discussion

The simultaneous expression of the two subunit genes COL4A1 and COL4A2 is essential for the formation of the heterotrimeric collagen IV molecule. The head-to-head arrangement of the two genes and their shared promoter appears to be particularly suitable to coordinate their expression. The shared promoter alone does not exhibit any transcriptional activity. However, we defined two activating elements located downstream of the transcription initiation sites of the COL4A1 and the COL4A2 genes, which were indispensable for even low levels of activity. The function of these two activating elements depends strongly on their relative position. Both elements are only functional when located downstream of the initiation site of transcription close to the shared promoter (see Fig. 1). Direct measurements of transcription initiation by nuclear run-off transcription experiments (see Fig. 3) revealed that the observed activities are mainly based on transcriptional stimulation and are not based

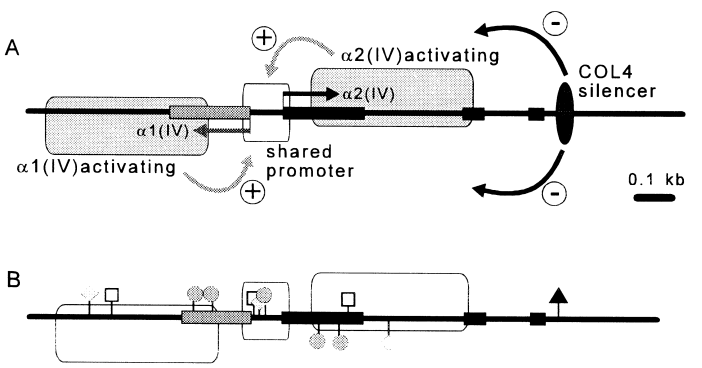


Fig. 4. Arrangement of regulatory elements of human collagen type IV genes. A: Localization of the shared promoter and both gene-specific activating elements as reviewed in [46]. We propose a model which is based on alternative, but mutually exclusive interactions of the gene-specific activating elements (arrows) with their shared promoter which define direction as well as efficiency of transcription. The recently characterized negatively acting COL4 silencer is able to inhibit the transcription of both genes [25]. B: The binding sites for Sp1 (circles), a CAAT-binding protein (squares) and CTCBF (rhombus) were defined previously [26] and their positions on different strands is indicated schematically. The factor SILBF (triangle) was shown to be essential for the function of the COL4 silencer [25].

on effects mRNA stability or processing as seen in some cases [34]. The distance of the activating elements from the shared promoter define them as separate elements different from promoter-distal sequences contacted by the basal transcription complex [37]. The two collagen type IV activating elements also do not resemble classical enhancers [33] due to their loss of function when placed far distant from the promoter (see Fig. 1B).

The murine COL4A1 and COL4A2 genes are organized in a similar transcriptional unit to the human genes [10,11], but distinct from the situation in man, an enhancer was identified within the first intron of the COL4A1 gene stimulating the transcription of both genes [10]. Yet, we were not able to detect a comparable enhancing element in the human genes, although large genomic regions were screened for such a function (R. Pollner, unpublished). The potential lack of a comparable element may reflect distinct species-specific constraints for the expression of these genes. Interestingly, the two genes coding for the equivalent of basement membrane specific collagen type IV in *C. elegans* are located on different chromosomes and therefore must be regulated in a different way [14].

Determination of the transcription rate in vivo revealed that the COL4A1 gene is transcribed more efficiently in vivo than the COL4A2 gene. An average ratio of about 2:1 was detected for HT1080 cells (Fig. 3B), a ratio reflecting the chain composition of the end product. Otherwise, the relative rates of divergent transcription were found to be similar in transient transfection experiments (see Figs. 1 and 2). Therefore, additional elements may be missing in the constructs used. Earlier experiments suggested the presence of an inhibitory element within the COL4A2 gene [8] which was mapped recently within the third intron of the COL4A2 gene and was suspected to counterbalance the effects of the activating regions under in vivo conditions [25].

The COL4A3-COL4A4 and COL4A5-COL4A6 genes are also arranged in divergently transcribed gene pairs, but only the promoter region of COL4A5-COL4A6 has been analyzed in more detail [38]. Two alternative promoters of the COL4A6 alternative mRNA species with different 5′-untranslated regions. The function of these transcripts and the involvement of regulatory elements for the control of expression have not yet been defined. The structures of collagen IV gene pairs are different from the situation of other collagens composed of different subunit chains, like collagen type I [39]. Although the two subunit genes are located on different chromosomes, genes are transcribed coordinately which is additionally reflected by the steady state levels of mRNAs [40]. In contrast, varying ratios of α1(IV) and α2(IV) mRNAs were detected in different tissues and cells indicating the involvement of both transcriptional and post-transcriptional regulatory events in expression of COL4 genes in vivo [35,36,41].

The sequence of the shared promoter exhibits a partial palindromic symmetry [8]. At least three distinct transacting factors, a CAAT-binding protein, Sp1 and a novel factor named CTCBF [26] interact with the promoter and mutational analysis revealed their involvement in transcriptional regulation. Based on these data we proposed that the shared promoter represents a region of two overlapping promoters with common elements with differential effects on gene-specific transcription [23]. Nevertheless, the promoter is functional in both directions when combined with a downstream activating element (Fig. 2). Binding of the same factors was detected within the activating elements of the COL4A1 as well as the COL4A2 genes [25]. The colocalization of cis-acting elements, overall arrangement of binding sites and the high degree of symmetry indicate the involvement of the bound factors in regulating the expression of the COL4 genes (see Fig. 4).

Current models for regulation of transcription initiation involve the specific binding of nuclear factors to regulatory sequences and their cooperative, synergistic or competitive effects on the basal transcriptional machinery [42,43]. The essential contacts among different factors are enabled either by their close association or by looping of intermediate regions. Based on these principles, we propose a model for the coordinated expression of collagen type IV genes based on the alternative interactions of the gene-specific activating elements with their shared promoter (Fig. 4). Either direct contacts or bridging cofactors induce looping and consecutively induce the directed initiation of transcription. A potential candidate for protein-protein contacts may be Sp1, which is able to form aggregates and stabilize loops of connecting DNA [44,45]. This model ('flip-flop loop') of alternative, mutually exclusive interactions may simply explain the reduced activity of constructs containing both activating elements (pXA, pANaSA) by their competitivity for the common promoter. The coordination of transcription may be based on the dissolution of gene-specific interactions by the elongating polymerase II and therefore the ability to induce new rounds of divergent transcription initiation.

The bidirectional transcription unit of the human collagen type IV genes represents a unique and specialized system for the coordinated expression of two closely linked genes and the data presented indicate that transcription is controlled by cooperative as well as competitive interactions of distinct regulatory elements.

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