Identification of a novel sequence element in the common promoter region of human collagen type IV genes, involved in the regulation of divergent transcription

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The expression of the heterotrimeric collagen IV molecule $\alpha 1(\text{IV})_2\alpha 2(\text{IV})$ is essential for the structural integrity and functional properties of all basement membranes. The two genes *COL4A1* and *COL4A2* that code for the subunits are found closely linked on chromosome 13 in a head-to-head arrangement and are transcribed in divergent directions. We have identified a novel *trans*-acting factor that binds *in vitro* to a unique homopyrimidine/homopurine stretch within the shared promoter region of the two collagen IV genes. Additional binding sites have been identified within the first introns of both genes

and the consensus sequence CCCTYCCCC for efficient binding has been deduced; the factor was named therefore 'CTC-binding factor' or 'CTCBF'. Mutations in the binding site of CTC-binding factor within the promoter inhibited binding *in vitro* and resulted in reduced transcription from both genes. The effect of mutations on the transcription of *COL4A2* is more pronounced than on the transcription of *COL4A1*. CTC-binding factor is a nuclear factor that binds dominantly *in vitro* to the collagen IV promoter and is involved in regulating the expression of both collagen IV genes.

INTRODUCTION

Collagen IV is the major structural component of all basement membranes in multicellular differentiated organisms [1,2]. The molecule is composed of two distinct subunits, the $\alpha 1(\text{IV})$ and the $\alpha 2(\text{IV})$ chains, which are both essential to form the functional heterotrimeric molecule $\alpha 1(\text{IV})_2\alpha 2(\text{IV})$ [3,4]. In the extracellular space these molecules form a complex network [5] in which other basement-membrane constituents, like laminin, nidogen and proteoglycans, are embedded [6]. In addition, type IV collagen is involved strongly in the interactions of basement membranes with cells [4]. The functional importance of collagen IV in the formation of basement membranes and in the organization of an organism is documented impressively by mutants of the collagen IV gene in *Caenorhabditis elegans*, which result in an embryonal lethal phenotype [7].

The human collagen IV genes have been isolated and show a complex organization [8,9] that differs from the typical 54 bp-repetition pattern that is present in the genes of fibrillar collagens [10,11]. Both human genes *COL4A1* and *COL4A2* are found closely linked on the distal long arm of chromosome 13 [12]. They are oriented head-to-head and are transcribed in opposite directions. The short sequence that separates the 5' ends of the two genes is 127 bp long and represents the common promoter region for both genes [13,14]. A similar organization was found for the mouse genes as well [15,16]. In contrast the two genes encoding the basement-membrane-specific collagen of *C. elegans* were found on different chromosomes [17]. The human promoter region alone showed no intrinsic transcriptional activity in transient transfection assays [13]. The transcription of both genes depends strongly on additional regions that are located downstream of the transcriptional start sites of either gene [18].

A similar organization of closely linked genes in a bi-directional transcription unit is found in some other loci, such as the *gall-*

gal10 [19] and histone *H2A-H2B* genes in yeast [20], *yp1-yp2* [21] and the *surfeit* locus [22] in *Drosophila* and in the viral genomes of simian virus-40 (SV40) [23] or adenovirus [24]. In all these examples there is no strict demand for synchronous transcription as is the case with the collagen IV genes. In some other cases, such as the genes for dihydrofolate reductase (DHFR) [25,26], insulin II of rat [27] and the murine thymidine kinase gene [28], opposite-strand transcription was detected in some instances, although the significance of these transcripts is not yet clear. To understand the mechanisms involved in the combined transcription of the collagen IV genes we have started to analyse the *cis*-acting elements involved and the nuclear factors' interaction with this region.

Here we describe the identification and characterization of a new nuclear factor involved in regulating the transcription of both human collagen IV genes. This factor is the dominating binding protein within the promoter *in vitro* and recognizes a homopyrimidine/purine sequence (the 'CTC box'). The factor was named therefore 'CTC-binding factor' or 'CTCBF'. The binding of CTCBF is necessary for the efficient transcription of both genes, although a preference for stimulating the transcription of *COL4A2* can be seen. Additional binding sites for CTCBF were detected within both genes, in a symmetrical arrangement relative to the central binding site within the promoter. This arrangement indicates their role in regulating the divergent transcription of human collagen IV genes.

MATERIALS AND METHODS

Oligonucleotides and fragments

The numbering of all oligonucleotides and fragments refers to the entry HSCOL4A12 in the EMBL database. Complementary oligonucleotides were synthesized (Applied Biosystems) with either a CG (upper strand) or a GATC protruding at the 5' end.

Abbreviations used: c, complex; CTCBF, CTC-binding factor; oligo, oligonucleotide.

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Oligonucleotides represent the following sequences: oligonucleotide 1, nt 6160–6183; oligonucleotide 2, nt 6179–6205; oligonucleotide 3, nt 6198–6225; and oligonucleotide 4, nt 6215–6239. Annealed oligonucleotides were either used directly or were subcloned, after treatment with Klenow polymerase, into the *SmaI* site of the vector pBSIISK⁺ (Stratagene) and inserts were isolated from these clones by cleaving with *Bam*HI and *Hind*III. The fragments were named oligo/BH. Fragments were isolated from genomic clones [13] by cleaving with the enzymes indicated. The following fragments were used: SaS (*SacI*–*SmaI*, nt 6166–6271); SS (*SmaI*–*SmaI*, nt 6120–6271); SN (*SacI*–*NcoI*, nt 5513–6014); EN (*EcoRI*–*NcoI*, nt 5703–6014); BM (*Bam*HI–*MaeI*, nt 6365–6588); MM (*MaeI*–*MaeI*, nt 6588–6845); MS (*MaeI*–*SacI*, nt 6588–6657); SM (*SacI*–*MaeI*, nt 6657–6845). MCS represents the polylinker region of pUC9.

Nuclear extracts and gel-retardation assay

HT1080 cells [29] were grown to confluency and nuclear extracts from 200–300 plates were prepared as described [30]. Extracts from HeLa cells were prepared exactly as described [31]. Extracts from Jurkat and EW cells were generous gifts from H. Klier, Martinsried. Gel retardation assays were carried out as described [32–34] with some minor variations. 2–3 µg of the nuclear extract was incubated with 0.1–6 µg of nonspecific competitors [poly(dA-dT), poly(dI-dC) or sonicated *Escherichia coli* DNA] for 10 min in 25 µl of buffer containing 25 mM Hepes/KOH (pH 7.5), and 50–250 mM NaCl. After adding 1–10 fmol 5′ end-labelled fragment, the reactions were kept at room temperature for 20 min and then were separated on 4% or 6% non-denaturing polyacrylamide gels in Tris/glycine or Tris/borate buffer. The gels were fixed and dried and the labelled bands were visualized by autoradiography. For competition experiments a 10–200-fold molar excess of the unlabelled fragments was added before adding the labelled fragment and was incubated for 10 min. The complex-formation constant was determined using the method of [35] and the amounts of the DNA–protein complex and the free fragment were determined after gel retardation by scintillation counting of the radioactivity in the corresponding bands.

U.v.-crosslinking experiments

Oligo2/BH was labelled at the 5′ end and 50 µl of the labelled oligonucleotide was used in standard binding reactions containing nuclear extracts from HT1080 cells. For competition experiments a 100-fold molar excess of unlabelled oligo1/BH or oligo2/BH was added to the pre-incubation mixture. The binding reactions were fractionated on a 1% low-gelling-temperature agarose gel in 0.5 × Tris/borate buffer, irradiated within the gel for 0–30 min with a germicidal u.v.-lamp at a distance of 12 cm, exposed and bands corresponding to CTCBF were cut out. The gel pieces were heated to 96 °C and aliquots were separated by SDS/PAGE (5–15%). Alternatively, the binding reactions were spotted onto Saran wrap, placed directly on an u.v.-lamp (254 nm) at 4 °C for 0–150 min and fractionated by SDS/PAGE. The gels were fixed, dried and exposed. Molecular weights were determined by comparison with parallel lanes containing ¹⁴C-labelled and prestained marker proteins.

Modification-interference assays

Methylation interference was carried out by the method of [36]. Fragment oligo2/BH was labelled at the 5′ end of the upper and

lower strands, whereas only the upper strand of the fragment MS was labelled. The labelled fragments were modified by dimethyl sulphate and were used in gel-retardation assays (ten-fold reaction) under conditions that showed no residual free fragment, when using non-modified fragments. The DNA was isolated from the bands representing the specific complex and the free fragment, treated with piperidine and was separated on sequencing gels, in parallel to base-specific sequencing reactions of the same fragments [37]. Some of the labelled fragments were also treated with hydroxyl radicals [38] and with DNAase I [39] and, after preparative gel-retardation assays, the DNA isolated from the specific complex and from the free fragment was separated on sequencing gels without further treatment.

Chimeric constructs and transient transfection

The construct pNA was generated by exchanging the herpes simplex virus thymidine kinase (HSV TK) promoter of the vector pBLCAT2 [40] for a 0.85 kb *NcoI*–*ApaI* fragment (nt 6022–6866) from p361 [13]. pBNA_{SA} contains a 0.73 kb *Bam*HI–*NaeI* fragment (*SphI*–*ApaI*; nt 5641–6370) fused to the fragment SA (nt 6739–6866) in the same vector. The *NcoI* site overlapping with the start site of translation of $\alpha 1$ (IV) was deleted by cutting with *NcoI* and treatment with T4 DNA polymerase. The short deletion generated (nt 6016–6021) was filled with a linker containing additional *PstI* and *SalI* restriction sites. The constructs pNA, pBNA_{SA} and pBLCAT2 (20 µg) were transfected, together with pL51 (5 µg), containing the *lacZ* gene under the control of the Rous sarcoma virus long terminal repeat (RSV LTR), into HT1080 cells as described previously [41]. The activity of chloramphenicol acetyltransferase was determined [42] and was standardized for β -galactosidase activity, as an internal standard for transfection efficiency.

In vitro mutagenesis

Mutants of the promoter were generated by the gapped-duplex method of site-directed mutagenesis [43]. The 0.35 kb fragment NB (see Figure 10), representing nt 6022–6270 and including the restriction sites *SalI* and *XbaI* from pBLCAT2 from pNA, was cloned into M13 mp9am16. Mutations were introduced at nt 6189–6190 by annealing with complementary oligonucleotides containing the mutant sequences and the linearized and single-stranded M13mp9revd86 vector. Instead of the wild-type sequence CC at nt 6189–6190 the mutants contain one of TA (mutant 11), TT (6) or TG (60). Clones were analysed by sequencing and the 0.35 kb *SalI*/(*NcoI*)–*Bam*HI fragments containing the mutant promoter sequences were introduced into pNA and pBNA_{SA}, resulting in the constructs pNA11, pNA6, pNA60, pBNA_{SA}11, pBNA_{SA}6 and pBNA_{SA}60. The fragments SS, corresponding to nt 6120–6271, were isolated from the mutant clones and were used as competitors in gel-retardation assays.

RESULTS

The genomic fragments containing the common-promoter region and the 5′ regions of COL4A1 and COL4A2 were isolated as described earlier [13]. The organization of the genes and the sequence of the promoter are shown in Figure 1. To detect nuclear factors binding to the promoter region we used the fragment SaS (Figure 1) in gel-retardation assays. Nuclear extracts were isolated from the human-fibrosarcoma cell line HT1080, which is known to express collagen IV [29]. Using non-specific competitors, such as *E. coli* DNA or poly(dA-dT), one major

Figure 1 Genomic organization of the promoter region of human collagen IV genes *COL4A1* and *COL4A2*

Exon 1 of *COL4A1* (open box) and three exons of *COL4A2* (hatched boxes) are indicated together with the direction of transcription of $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ transcripts (arrows) and the partial sequence of the promoter. The fragments, sequence motifs and the position of the oligonucleotides used in the gel-retardation experiments are designated. The numbering refers to the entry HSCOL4A12 in the EMBL database.

complex can be detected (Figure 2a, lanes 1–6). The prominent band could be competed out by adding a molar excess of the unlabelled fragment SS, which includes the fragment SaS, but not by non-related vector sequences (MCS), which proves that the complex is specific (Figure 2a, lanes 7–11). The minor, faster migrating complexes observed with some of the extracts used appear to represent non-specific complexes, since no competition can be seen with the fragment SS. To localize the binding site of the nuclear factor in this dominantly formed complex, we used the overlapping oligonucleotides 1, 2, 3 and 4 (see Figure 1), from the *COL4A1* proximal half of the promoter, in competition assays. Only oligonucleotide 2 could compete to form the dominant complex on the promoter (Figure 2a, lanes 12–16).

To substantiate the binding of the nuclear factor to this sequence further, we used the subcloned oligonucleotide 2 (oligo 2/BH) in gel-retardation assays (Figure 2b). Only one complex was detected and its specificity was demonstrated by competition with an excess of the homologous oligo 2/BH and the promoter fragment SS. No competition was observed with the oligonucleotides 1 and 3, representing the CCAAT motif and the GC box, respectively, nor with other non-related vector sequences MCS. These data indicated that one major protein–DNA complex forms on a sequence resembling oligo 2 within the common-promoter region of the two collagen IV genes, under the experimental conditions used.

To determine the binding site of this nuclear factor, different modification assays were carried out. For methylation interference [36], oligo2/BH was labelled on the 5' end of the 'upper' and of the 'lower' strand. After partial methylation and gel retardation, the DNA from the retarded DNA–protein complex and the free fragment was isolated and was analysed on sequencing gels (Figures 3a and 3b). The methylation of guanine residues in the lower strand within the sequence 5'-GGGG-GAGGGG (nt 6196–6187) inhibited the interaction with the factor, whereas no interference was detected with methylated guanines in the upper strand. Since methylation interference does not allow the involvement of cytosine residues in the interaction

with the protein to be determined, the 5' end labelled oligo2/BH was pretreated with DNAase I [39] and hydroxyl radicals [38]. The analysis of these samples (Figures 3c and 3d) revealed that nicking of the DNA interfered with binding only within the pyrimidine-rich sequence. This sequence is remarkably sensitive to digestion with DNAase I as shown in Figure 3(c). The results of the modification–interference assays are summarized in Figure 3(e) and define the binding site of this nuclear factor within the homopyrimidine/purine stretch of the promoter and prove that the factor interacts with both strands. We named this motif the 'CTC box' from the sequence C_5TC_7 , to which it binds and the interacting factor 'CTC-binding factor' or 'CTCBF'.

The characteristics for efficient binding of CTCBF were analysed in gel-retardation assays using the fragment oligo2/BH. Non-specific binding can be diminished by adding the homopolymers poly(dA–dT), poly(dI–dC) or sonicated *E. coli* DNA (results not shown). Maximal binding was detected for 0.08 $\mu\text{g}/\text{ml}$ poly(dA–dT) or poly(dI–dC), whereas sonicated *E. coli* DNA slightly reduced the amount of specific complex, presumably due the higher complexity of *E. coli* sequences.

The binding of CTCBF can be detected within a range 50–300 mM NaCl, with concentrations of 125–200 mM being most efficient (Figure 4a). Concentrations exceeding 300 mM prevented binding. Therefore an optimal concentration of 150 mM was added in most experiments. Similar results were seen when KCl was used instead of NaCl (results not shown). The binding of CTCBF can be detected at 4 °C, but is optimal at 20–30 °C. Temperatures higher than 40 °C destroy the DNA-binding ability of CTCBF. The formation of the complex seems to be very fast, since binding of CTCBF can be detected as early as after 15 s of incubation. Complex formation is completed after 10 min and remains stable for at least 3 h (results not shown). Incubation was carried out routinely for 20 min at room temperature in a buffer containing 150 mM NaCl and 0.08 $\mu\text{g}/\text{ml}$ poly(dA–dT).

The binding of CTCBF is highly sensitive to adding EDTA (2–10 mM) and *o*-phenanthroline, but is insensitive to EGTA

Figure 2 The human collagen IV promoter interacts *in vitro* preferentially with one nuclear factor

(a) Gel-retardation assay using the labelled fragment SaS and 2 μ g of nuclear extract from HT1080 cells, together with 1, 2 and 4 μ g of poly(dA-dT) (lanes 1–3) or 0.5, 1 and 2 μ g of *E. coli* DNA (lanes 4–6). Competition assays were done after adding zero (lanes 7, 16), 100-, 200- or 400-fold molar excess of the unlabelled fragment SS (lanes 8–10), a 200-fold molar excess of the polylinker fragment MCS from pUC9 (lane 11) or oligonucleotides 1–4 (lanes 12–15). The specific complex is indicated by an arrow. (b) Gel-retardation assay of the labelled fragment oligo2/BH. All the reactions included 2 μ g of poly(dA-dT), together with zero (—) (lane 3), a 100-fold (lanes 2 and 4) or 200-fold molar excess (lanes 1 and 5) of the fragments oligo2/BH (lanes 1 and 2) and SS (lanes 4 and 5), and a 100-fold molar excess of oligo1/BH (lane 6) or of MCS (lane 7).

(Figure 4b). The inhibitory effect of EDTA can be reversed by adding equimolar amounts of Zn^{2+} ions (Figure 4c). Whether this effect indicates the involvement of zinc fingers in the DNA-binding domain of CTCBF has to be determined by the purification and characterization of the factor.

The equilibrium-binding constant of CTCBF was determined [35] for the binding site within the promoter (Figure 5). As a first step, the concentration of CTCBF in the nuclear extract from HT 1080 cells was determined by gel-retardation assays, using

Figure 3 Determination of the CTCBF-binding site within oligo2/BH by modification-interference assays

The orientation of the strands refers to Figure 1. **(a), (b)** Methylation-interference assays of oligo2/BH. The fragment was labelled either at the 5' end of the lower **(a)** or the upper strand **(b)**, partially methylated by dimethyl sulphate (DMS) and used in gel-retardation experiments. The free fragment (lane 0) or the CTCBF–DNA complex (lane 1) were isolated, treated with piperidine and separated on a sequencing gel, parallel with sequencing reactions specific for G or G + A (lanes G, G + A). The positions of interference by methylation are indicated by asterisks. **(c), (d)** Determination of the interference of CTCBF binding with the pyrimidine-rich upper strand after pretreatment with DNase I **(c)** or hydroxyl radicals **(d)**. The DNA was isolated from the free fragment (lane 0) and from the CTCBF–DNA complex (lane 1) and was analysed directly on a sequencing gel together with a G-specific reaction (lane G) and the pretreated fragment before the binding reaction (lane K). The regions of interference are indicated. **(e)** Summary of the interference data.

conditions with constant, but limiting, amounts of the factor and increasing concentrations of the labelled oligo 2/BH of known specific activity. The molar amount of the factor was calculated after determining the radioactivity in the retarded DNA-protein bands. Assuming that only one CTCBF molecule is bound to oligo2/BH, a concentration of 1.5 fmol of CTCBF/ μ g of total protein was determined for the nuclear extract used in these experiments (results not shown). As a second step, the complex-formation constant was calculated from the ratios of the free fragment and the retarded DNA-protein complex, after gel-retardation assays under equilibrium conditions. Figure 5(a) shows a typical experiment where equal amounts of CTCBF (c° 0.11 fmol) were incubated with increasing concentrations of the labelled oligo2/BH (DNA $^{\circ}$ 0.07–1 fmol), separated by gel retardation and the amounts of complex (c) and free oligo2/BH (DNA) were measured. For the CTC motif in the human collagen IV promoter, represented by oligo2/BH, we determined a mean value for the complex-formation constant of $4 \times 10^9 \text{ M}^{-1}$ (Figure 5b). This value indicates that CTCBF binds with medium affinity to its recognition sequence, compared with non-specific interactions of DNA-binding proteins (K $5 \times 10^6 \text{ M}^{-1}$) or a high-

Figure 4 Determination of the binding characteristics of CTCBF by gel-retardation assays using the labelled fragment oligo2/BH

(a) Determination of the optimal concentration of NaCl for binding to CTCBF by adding 50–200 mM NaCl (lanes 1–7). (b) The binding of CTCBF (—, lane 11) is highly sensitive to the presence of 10 mM EDTA (lane 8) and *o*-phenanthroline (lane 10), but not to EGTA (lane 9). (c) Complex formation (lane 12) can be inhibited by the addition of 2 mM EDTA (lane 13). The effect can be reversed by adding equimolar amounts of $ZnCl_2$ (lane 14).

Figure 5 Determination of the complex-formation constant of CTCBF for the CTC-box motif

(a) Gel-retardation assay of constant amounts of nuclear extracts that had been incubated with 0.07–1 fmol of the labelled fragment oligo2/BH (DNA^0). The concentration of CTCBF in a nuclear extract from HT1080 cells was determined by titration with known amounts of labelled oligo2/BH ($c^0 = 0.11$ fmol). The CTCBF–oligo2/BH complex and the free fragment were cut out and the radioactivity was measured. (b) The complex-formation constant was calculated from the concentrations of CTCBF complex $[c]$ and unbound fragment $[DNA]$.

affinity binding factor like nuclear factor-I (NFI) ($K \times 10^{11} M^{-1}$) [35].

Motifs similar to the CTC box of the promoter region were found within both collagen IV genes as well by sequence comparison, as shown in Figure 6(a) and listed in Table 1. To test for the binding of CTCBF to these sequences, the fragments EN and SN from *COL4A1* and BM, MM, MS and SM, corresponding to the *COL4A2* gene, were used as competitors in gel-

Figure 6 Detection of additional binding sites for CTCBF within both genes by competition with different fragments in gel-retardation assays using the labelled fragment oligo2/BH

(a) Map of the collagen IV genes (see Figure 1), position of restriction sites and the fragments used for competition. Binding sites with high (1, 2 and 4) and low (3) affinity are marked by black diamonds. Restriction sites are M, *MaeI*; B, *BamHI*; E, *EcoRI*; S, *SacI*; Sm, *SmaI* and N, *NcoI*. (b) Gel retardation assays using either zero (—), 50-, 100- or 200-fold molar excess of the fragments indicated.

retardation assays (Figure 6b). Fragment SN, but not EN, competed strongly for interaction with CTCBF (Figure 6b, lanes 2–4, 6–8) indicating a binding site within the first intron of *COL4A1* (Figure 6a, site 1). Competition with the fragments MM, MS and BM defined a high-affinity binding site within fragment MS (Figure 6b, lanes 14–16 and 18–20) and a low-affinity binding site within fragment BM (Figure 6b, lanes 10–12), resembling motifs within the first intron and exon of *COL4A2*, respectively (Figure 6a, sites 3 and 4). Fragment SM, which does not contain a CTC-box-related sequence, did not compete for the binding of CTCBF (Figure 6b, lanes 22–24). These results for the interaction of CTCBF with the binding sites 1, 3 and 4 (Figure 6a) were confirmed by gel-retardation assays using the fragments SN, MS and BM as labelled probes and by modification-interference assays (data not shown).

The consensus sequence for the efficient binding of CTCBF can be deduced by comparing all the binding sites as shown in Table 1. All the recognition sequences contain the sequence 5'-CCCTYCCc as a common motif. When the two 3' terminal C residues (in italics) are changed, as seen with the low-affinity

Table 1 Binding sites for CTCBF

Sites within the human collagen IV genes are numbered according to Figure 6(b) and the position and relative orientation refer to the EMBL database, accession number HSCOL4A12. Other sequences are taken from the references cited.

Site	Location	Position	Orientation	Sequence	Affinity
1	<i>COL4A1</i> /intron1	5691–5699	>	CCCTTCCCC	High
2	Promoter	6191–6196	>	CCCTCCCCC	High
3	<i>COL4A2</i> /exon1	6482–6466	<	CCCTTCAG	Low
4	<i>COL4A2</i> /intron1	6635–6619	<	CCCTCCCCC	High
Consensus				CCCTYCCC	
Adenovirus/ <i>I</i> Va2 [24]		– 54 – 38		ACCCCTCCCACT	–
<i>D. discoideum</i> / α -fucosidase [34]		– 271 – 286		CCCCACCCCTA	–

Figure 7 Detection of the factor CTCBF in nuclear extracts from different cells

Gel-retardation assays using labelled oligo2/BH and 2 μ g of nuclear extracts from HT1080, HeLa, Jurkat and EW cells. Reaction contained zero (–) or a 100-fold molar excess (+) of oligo2/BH as the competitor.

binding site 3 in the *COL4A2* gene, some affinity for binding CTCBF is lost, but the specificity is still retained.

Motifs similar to the deduced consensus sequence can be found in some other genes. CTC-box-related sequences have been found close to the adenovirus *I*Va2 gene [24] and within the 'G-box' of the α -fucosidase gene of *Dictyostelium discoideum* [44]. Oligonucleotides resembling these sequences competed for the binding of CTCBF (data not shown). Whether CTCBF or related factors with similar binding characteristics interact with these motifs is not yet clear. A negatively acting factor IF1 was described recently that binds to a GC-rich sequence in the promoter of the *COL1A1* [45]. However oligonucleotides specific for binding this factor were not able to compete for the binding of CTCBF to oligo2/BH, excluding the possibility that the two factors are identical (data not shown).

The CTC-binding factor was identified first in nuclear extracts from HT1080 cells, which are known to produce collagen IV (Figure 2). Whether the appearance of CTCBF correlates with cell-specific production of collagen IV was tested by gel-retardation assays using nuclear extracts from different cell lines

(Figure 7). Collagen IV is produced by the human-fibrosarcoma line HT1080 and in low amounts by HeLa cells [29], whereas the T-lymphocyte Jurkat and the B-lymphocyte EW cell lines do not synthesize basement-membrane components. Complexes resembling CTCBF could be detected in all extracts tested and the specificity of interaction was shown by competition with oligo 2 (Figure 7, lanes 2, 4 and 7). The same CTCBF-specific complex can also be detected in extracts of placenta (data not shown). The expression of CTCBF is obviously not restricted to collagen IV-producing cells and therefore CTCBF represents a ubiquitous DNA-binding protein and not a tissue-specific nuclear factor. The highly restricted expression of collagen type IV genes must be regulated by additional factors or by other mechanisms.

Attempts to fractionate nuclear extracts from HT1080 and HeLa cells by chromatographic methods showed that CTCBF is very unstable during purification and therefore the amounts of purified factor obtained were not sufficient to characterize the protein (data not shown). We used u.v.-crosslinking studies as an alternative approach to determine the size of proteins bound specifically to labelled oligo2/BH (Figure 8). Irradiation of CTCBF-specific complexes by u.v. within the gel (Figure 8a) or in solution (Figure 8b) resulted in the transfer of label to proteins of an apparent molecular weight of 75 kDa and of about 110 kDa. In some experiments the 75-kDa protein could be resolved into at least two closely spaced bands (Figure 8a, lanes 2 and 3), that may represent either two different proteins or variable forms of the same protein. The 110-kDa protein can be detected only after prolonged exposure to u.v. (Figure 8a, lanes 4 and 6). The crosslinking reaction can be competed specifically by adding an excess of unlabelled oligo2/BH (Figure 8a, lane 5; 8b, lanes 4, 7 and 10), but not the unrelated oligo1/BH (Figure 8b, lanes 5, 8 and 11). Therefore the 75-kDa and 110-kDa crosslinkage products represent proteins that are involved in the specific interaction of CTCBF with DNA. It is not possible to define the molecular structure of the specific CTCBF complex based on these data. The 75-kDa protein at least is involved in the interaction with DNA. Whether this protein binds in the form of a homodimer or whether additional proteins are involved in forming the complex can be decided only by characterizing the purified CTCBF complex.

The functional importance of bound CTCBF was tested after mutation of the CTC motif within the promoter region. The central positions of the CTC motif CCCTCCC in the promoter were changed to CTATCCC (mutant 11), CTTTCCC (6) or CTGTCCC (60) by the gapped-duplex method [18] within the context of the fragment NB (see Figure 10 and Table 2). The promoter fragment SS (see Figure 6a) from the wild-type and from the different mutants were tested in gel-retardation assays

Figure 8 Identification of DNA-binding proteins of the CTCBF complex by u.v.-crosslinking

The binding reactions contained labelled oligo2/BH and nuclear extracts from HT1080 cells and u.v.-crosslinking was done by two methods. (a) The binding reactions were fractionated in a 1% low-gelling-temperature agarose gel, irradiated with u.v. for the times indicated, exposed and CTCBF-specific bands were isolated. (b) The binding reactions were irradiated after direct spotting onto a u.v.-lamp (254 nm) at 4 °C for the times indicated. Competition reactions were performed by adding a 100-fold molar excess of the unlabelled oligo1/BH (b, lanes 5, 9 and 11) or oligo2/BH (a, lane 5; b, lanes 4, 7 and 10). Proteins were separated by SDS/PAGE and the labelled proteins were visualized by autoradiography. Positions of specific crosslink products (arrows) and the positions of molecular-mass marker proteins (lane M) are indicated.

Figure 9 Mutagenesis of the CTC box eliminates the binding of CTCBF

Gel-retardation assays of the labelled fragment oligo2/BH with zero (lane 3), 100-fold (lanes 2, 4, 6, 8 and 10) or 200-fold (lanes 1, 5, 7, 9 and 11) molar excess of oligo2/BH (lanes 1 and 2), oligo 1/BH (lane 12) or fragments SS from the wild-type (wt) or different mutant (lanes 4-11). The sequence of the mutants is shown in Figure 10.

for their ability to compete for the binding of CTCBF (Figure 9). In contrast with the wild-type (wt) fragment SS^{wt}, all the mutated promoter fragments SS¹¹, SS⁶ and SS⁶⁰ could not compete for the binding of CTCBF to the fragment oligo2/BH. Therefore the affinity for CTCBF was strongly reduced in all the mutated CTC-box sequences.

To compare the transcriptional activity of the mutated promoters, they were inserted into constructs that can detect transcription either in the $\alpha 1(\text{IV})$ or in the $\alpha 2(\text{IV})$ direction. It was shown earlier that the common promoter is inactive and that sequences downstream from both genes are necessary for efficient

Figure 10 Mutagenesis of the CTC box

The constructs pNA and pBNA5A are shown in parallel with the genomic organization and the CTC-box motif in the promoter is indicated (black rhombus). Arrows indicate the direction of transcription and ATG scored through indicates the deletion of the translational initiation signal of *COL4A1*.

Table 2 Effect of mutagenesis of the CTC box on the transcription of *COL4A1* and *COL4A2*

The effect of the mutagenesis of the CTC-box motif on the transcription of the *COL4A1* and *COL4A2* genes, as seen in the constructs pNA and pBNA5A. The sequence (nt 6186-6198) of the wild-type and different mutants (6, 11 and 60) is shown and the residual activity of the mutant constructs is expressed relative to the wild-type constructs, which is set at 100%.

Mutant	Sequence	Expression (%)	
		$\alpha 1(\text{IV})$ pBNA5A ^{mut}	$\alpha 2(\text{IV})$ pNA ^{mut}
Wild-type	CCCCCTCCCCCC	100	100
11	CCCTATCCCCCC	82	30
6	CCCTTTCCCCCC	63	35
60	CCCTCTCCCCCC	56	35

gene-specific transcription. Therefore construct pNA, used to analyse transcription in the $\alpha 2(\text{IV})$ direction, contained intron 1 and exon 2 of *COL4A2* linked to the chloramphenicol acetyltransferase (CAT) gene [18], whereas pBNA5A, used to detect transcription in the opposite direction, contained additional sequences from the first intron of *COL4A1* and a splice-acceptor site for proper processing (Figure 10). The translational start site within exon 1 of *COL4A1* in pBNA5A was deleted (see Materials and methods). The wild-type and mutated promoter fragments, as represented by the fragment NB, were included in both constructs in the proper orientation, resulting in the constructs pNA11, pNA6 and pNA60 and the respective pBNA5A variants. To measure the effects of these mutations on transcriptional activity, the constructs were transfected into HT1080 cells and the chloramphenicol acetyltransferase activity was determined. All the mutant constructs had decreased levels of activity when compared with the wild-type constructs pNA and pBNA5A (Table 2). Mutations within the CTC box never caused a complete loss of activity, which suggests that co-operative effects with other factors are involved in regulating the *COL4* genes. It was striking that mutations of the CTC box had a significantly stronger influence on the transcription of the *COL4A2* gene (30-36% of the wild-type activity) than on the transcription of *COL4A1* (56-82%). These experiments prove that CTCBF is essential for efficient transcription of the *COL4A2* and *COL4A1* genes, although the different effects of the

mutations on the transcription in either direction indicate a preference for stimulating *COL4A2* expression.

DISCUSSION

We have demonstrated that a new nuclear factor binds specifically to the bi-directional collagen IV promoter region within a homopyrimidine/purine stretch (the 'CTC box') and the factor was named therefore the 'CTC-box binding factor' or 'CTCBF'. Mutagenesis of the CTC box prevented the binding of CTCBF *in vitro* and resulted in a strong decrease in the transcription of both genes when tested by transient-transfection assays. Therefore CTCBF is involved in the transcriptional control of both genes. This factor recognizes also additional CTC-box motifs located within the first introns of both genes. The symmetrical arrangement of the binding sites for CTCBF may be important for the co-ordination of bi-directional transcription of the human collagen IV genes.

The interaction of CTCBF with the CTC-box motif in the promoter region of the collagen IV genes was identified by gel-retardation assays and it was shown to interact with both strands by modification-interference analysis. The consensus sequence CCCTCCc for the efficient binding of CTCBF was deduced by comparing different binding sites within both collagen IV genes (see Table 1) and agreed well with the data from modification analysis and from mutagenesis. Although the factor interacts with both strands of the DNA, we chose the name 'CTC-binding factor' because of its preference for stimulating the transcription of the $\alpha 2(\text{IV})$ gene. The CTC-box motif is found upstream on the same strand as the initiation site of the *COL4A2* gene (see Figure 1).

Recently a region overlapping with a CTC motif within the mouse collagen type IV promoter was shown by DNAase-footprinting analysis to be recognized by a nuclear factor from mouse engelbreth-holm-swarm (EHS) tumour extracts [46]. However the gel-retardation data differ significantly from our data with the human CTCBF. Whether this factor represents the murine homologue of the human CTCBF awaits the further characterization of this protein. A number of known nuclear factors recognize sequences similar to the CTC box [47] and we tested the binding motifs of Sp1 [48], GCF [49], AP2 [50] and ETF [51] for their ability to compete with the binding of CTCBF to the CTC box. Inhibition by any of these factors was not observed and so the possibility of the identity of CTCBF with these factors could be excluded. Sequences similar to the CTC motif are present in the *D. discoideum* α -fucosidase gene [44] and close to the *IVa2* gene of adenovirus [24], both of which exhibit a distinct affinity for CTCBF (data not shown). No clear functions have been defined for these sites yet, but it is suspected that these sequences are involved in the transcriptional regulation of these genes.

However it is striking that motifs comparable to the CTC box can be found close to the start sites of transcription in a number of genes coding for extracellular matrix genes, such as the *COL1A1* genes of human, rat and chick [52,53], the mouse and human laminin B2 genes [54,55] and the osteonectin genes from various species [56,57]. In the chicken *COL2A1* gene sites similar to the CTC box were found to be sensitive to S1 nuclease [58]. A similar sensitivity to nucleases could be seen as well with the CTC-box motif after digestion with DNAaseI (see Figure 3c), which suggests that this sequence may exhibit a different DNA structure as well. No function has been ascribed to these sites yet. Whether CTCBF can interact with these sequences is not yet clear, but the presence of similar sequence motifs in genes coding

for different extracellular matrix proteins implies that these elements may be important for the transcriptional control of all these genes.

CTCBF is not only present in human cells which synthesize collagen IV, such as the fibrosarcoma line HT1080 and HeLa cells [29], but is present in the lymphocyte cell lines EW and Jurkat that do not express collagen IV. This makes it unlikely that CTCBF alone is responsible for the tissue-specific expression of the collagen IV genes. Nevertheless the interaction with CTCBF is necessary for the efficient transcription of collagen type IV genes. Tissue-specific transcription may require the interaction of as yet unknown tissue-specific nuclear factors or may be regulated by other mechanisms, such as changes in DNA methylation or in chromatin structure. Differences in the pattern of CpG methylation were found in the collagen IV genes of mouse between undifferentiated and differentiated F9 cells [59]. Additionally *in vitro* methylation of constructs showing transcription of human collagen IV genes completely inhibits their transcriptional activity in transient-transfection assays (data not shown).

Binding of CTCBF to its recognition sequence is highly sensitive to the presence of EDTA and *o*-phenanthroline, but not to EGTA. The sensitivity to *o*-phenanthroline, which binds Zn^{2+} ions preferentially in the presence of other divalent cations, and the reversal of the inhibitory effect of EDTA by the addition of Zn^{2+} ions point to the idea that binding of CTCBF depends strongly on the availability of Zn^{2+} ions. Whether or not CTCBF contains Zn fingers cannot be decided yet.

U.v.-crosslinking data indicate that a protein of about 75 kDa is involved in DNA-binding with the CTCBF complex. The detection of an additional 110 kDa protein after longer exposure to u.v. may be due to the interaction of the 75 kDa protein as a homodimer or to the involvement of an additional protein. Because of the instability of CTCBF during chromatographic purification steps it has not been possible so far to purify sufficient amounts of the factor to characterize the potential subunit proteins of CTCBF (data not shown). These experiments indicate that CTCBF does not consist of a single protein, but rather may represent a multimeric complex of proteins that contains a 75 kDa protein at least. The purification of the factor is necessary therefore to characterize CTCBF in detail.

Mutation of the CTC-box proved the functional importance of CTCBF for the transcription of both human collagen IV genes. However it is striking that mutation of the binding site influenced the transcription of *COL4A2* more strongly than that of *COL4A1*. Additionally a comparable disproportionate effect on transcription in different directions was seen after mutations of the CCAAT motif and a GC box within the collagen IV promoter [62]. Therefore we assume that the common-promoter element of human collagen IV genes should not be understood as a common bi-directional element, but may be explained better by the idea of two overlapping, but functionally uni-directional, promoters with shared elements.

The interaction of CTCBF with additional CTC motifs within both genes was detected by competition experiments and binding studies and was proved by modification-interference assays. The arrangement of sites reveals a striking symmetry, relative to the central CTC box in the promoter (see Figure 6a). The motifs within both genes are found on different strands and therefore define a 'palindromic' arrangement, relative to the binding site within the promoter. A similar symmetrical and palindromic arrangement of sequences can be seen as well with the CCAAT and GC box motifs and even the promoter itself represents a palindromic element [13]. These binding sites co-localize with functional elements within both genes that have been found to be necessary for efficient transcription [18]. The highly symmetrical

arrangement of the binding sites stimulates speculation on the importance of this special organization for the divergent transcription of *COL4A1* and *COL4A2*. The binding of identical factors, CTCBF, a CCAAT-binding protein and Sp1, to three distinct regions, the shared promoter and elements located downstream within each gene, may enable direct or indirect interactions between the factors. Recently such interactions have been demonstrated for multiple bound Sp1 [60,61]. Some experiments with partially purified CTCBF support this hypothesis, because of its ability to form aggregates *in vitro* (G. Fischer, unpublished work). The idea of interactions between downstream elements with the shared promoter would implicate competitive effects between the two divergent transcription units, that have been seen in some constructs, in enabling divergent transcription of both genes [18]. Further analysis of the nuclear factors binding to the promoter and to additional regions and of their reciprocal interactions will allow insights into the mechanism of the bi-directional transcription of the human collagen IV genes.

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