c-Krox Binds to Several Sites in the Promoter of Both Mouse Type I Collagen Genes

STRUCTURE/FUNCTION STUDY AND DEVELOPMENTAL EXPRESSION ANALYSIS*

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We have previously shown that c-Krox is a zinc finger protein that can increase the transcriptional activity of the mouse α1(I) collagen promoter through its binding to two GC-rich sequences (Galéra, P., Musso, M., Ducy, P., and Karsenty, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9372–9376). In this report we show that c-Krox can bind to an additional site in the promoter of the α1(I) collagen gene and to three sites in the promoter of the α2(I) collagen gene, the other gene coding for type I collagen. One of the binding sites present in both promoters is adjacent to the CCAAT box. We have performed a structure/function analysis of c-Krox locating the transactivation domain in the zinc finger and C-terminal domains and the dimerization domain in the C-terminal end of the protein. We also demonstrate that c-Krox is an early response gene, whose expression is detectable as early as 9.5-day postcoitum in mouse embryos. Whole-mount in situ hybridization shows that c-Krox is expressed in dermatomes, the somite derivatives that generate dermis, and section in situ hybridization shows that c-Krox and αI(I) collagen mRNAs colocalized in skin but not in bone during development. This result is consistent with the predominant expression of c-Krox in skin in postnatal life. Thus, our findings suggest that c-Krox is one transcription factor controlling the coordinated expression of the two type I collagen genes in skin.

Type I collagen, a major constituent of the extracellular matrix of skin and bone, is synthesized mostly by two cell types, the fibroblast and the osteoblast (1). Type I collagen is a heteromeric protein composed of two α1(I) chains and one α2(I) chain that are encoded by two different genes, the α1(I) and α2(I) collagen genes (2). Numerous experiments using cultured fibroblasts have shown that in most circumstances the two polypeptide chains of type I collagen are synthesized with a 2:1 stoichiometry (3). The same 2:1 stoichiometry is also observed for the steady state levels of the corresponding mRNAs in human, mouse, and chick fibroblasts and for their rates of synthesis in nuclear run-on experiments with chick fibroblasts nuclei (4, 5) indicating that, in many instances, the expression of the α1(I) and α2(I) collagen genes is coordinately regulated at the transcriptional level. This coordinated synthesis of the two chains is critical for proper vertebrate development; furthermore, the synthesis of both chains is coordinately increased in several fibrotic conditions (6–8). Thus, it is important to understand the molecular mechanisms of the coordinated regulation of expression of these two genes for biological as well as medical purposes.

One hypothesis to explain how this coregulation of expression occurs predicts that common transcription factors are binding to identical cis-acting elements in the promoter of both genes. This hypothesis has been supported by the finding that two well characterized activators of transcription, Sp1 (9–11) and CBF (12–14), bind to the promoter elements of both the α1(I) and α2(I) collagen genes. Our previous studies on the mouse α1(I) collagen gene have identified several different cis-acting elements upstream of the start site of transcription (13). One of these elements containing a GC-rich sequence is present twice in this promoter, between −190 to −170 and −160 to −130. The two copies of this GC-rich element were called A α1(I) for the most distal and B α1(I) for the most proximal (13). Recently, we cloned a cDNA encoding a transcription factor called c-Krox that binds specifically to these two GC-rich elements (15). This GC-rich element is also present at approximately the same location in the promoter of the mouse α2(I) collagen gene, and we have shown previously that an identical binding activity, present in nuclear extracts of NIH3T3 mouse fibroblasts, bound to these three GC-rich sites in the α1(I) and in the α2(I) collagen promoters (14). This result suggests that c-Krox could bind also to the α2(I) collagen promoter.

c-Krox is preferentially expressed in skin, one of the major sites of type I collagen synthesis in adult animals, and is not expressed in bone (15). c-Krox belongs to a family of developmentally regulated genes conserved between Drosocephila and human. The first member of this gene family to be identified was Krüppel, a segmentation gene in Drosocephila (16). To date more than 20 mouse homologues of Krüppel, called the Krox genes, have been isolated as well as human homologues such as YY1 and GLI (17–19). These genes are early response genes that are transiently activated by serum or growth factors, and several of them, such as Krox 20 and Krox 24, are expressed in a tissue-specific manner (20, 21). These characteristics, along with the predominant expression of c-Krox in skin, raise the
possibility that c-Krox participates in the control of the type I collagen gene expression in skin.

Our long term goal is to understand the function of c-Krox in vivo. A preliminary step in this analysis is to show whether c-Krox binds to the promoter elements of both type I collagen genes and to identify the different functional domains of c-Krox. In this study we show that c-Krox binds to the promoter of both type I collagen genes. We also present the results of our structure/function analysis showing that c-Krox acts as an activator of transcription and has the ability to dimerize. Finally, we report that c-Krox is expressed in the dermatomes, which generate the dorsolateral dermis of adult animals and co-localized with α1(I) collagen expression in developing skin. Taken together our data suggest that c-Krox could be involved in the coordinated regulation of expression of the type I collagen genes in skin.

MATERIALS AND METHODS

DNA Constructions—Plasmids pAZ1003 and pG60 have been previously described (13, 22). Full-length GAL4-c-Krox was obtained by doing a 1.45-kilobase NaeI-XbaI fragment of the c-Krox cDNA into the Smal-XbaI site of pSG424 (23). GAL4 Δc-Krox (N), which contains the N-terminal domain of c-Krox, was generated by cloning a 525-bp NaeI-SmaI fragment of c-Krox into the NaeI-SmaI site of pSG424. GAL4 Δc-Krox (N + Zn) contains the N-terminal and zinc finger domains of c-Krox; it was generated by cloning a 987-bp NheI-SalI fragment of c-Krox into the NheI-SalI site of pSG424. GAL4 Δc-Krox (Zn), which contains the zinc finger domain of c-Krox; it was generated by cloning a 930-bp NheI-SalI fragment of c-Krox into the NheI-SalI site of pSG424. GAL4 Δc-Krox (C) contains the C-terminal domain; it was obtained by digesting the c-Krox cDNA with BsrMI, blunt-ended, digesting with XbaI and ligating this insert into pSG424 digested with Smal-XbaI. All constructs were verified by DNA sequencing. The CAT reporter plasmid used contains four copies of the GAL4 recognition sequence (5’-CTAGGGAGAATCT/CTCCTGGTG-3’) (24).

Generation of Histidine-c-Krox Fusion Protein, Nuclear Extract Preparation, and DNA Binding Assays—Full-length c-Krox and deletion mutants of c-Krox were expressed in Escherichia coli (E. coli) as fusion proteins with a histidine tag (His-tagged c-Krox) (25). An EcoRI fragment encoding the entire c-Krox cDNA done (15) was ligated in frame with coding sequences for the histidine tag (His-tagged c-Krox) (25). The purity of the expressed proteins was verified by DNA sequencing. Synthesis of the histidine fusion proteins was performed as described previously (15). The purity of the eluted proteins was verified on 10% SDS-PAGE followed by Coomassie Blue staining. Renaturation of c-Krox proteins was performed by dialyzing for 1 h against the c-Krox binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM dithiotreitol, 100 mM NaCl, 0.05 mg/ml bovine serum albumin, 0.1% Nonidet P-40, 10% glycerol, 25 μM ZnCl2) containing 2 M urea, followed by two dialyses of 1 h each against the same binding buffer containing 0.2 M urea and no urea, respectively. The amount of protein was measured by the Bio-Rad protein assay system. Nuclear extracts were prepared according to Dignam et al. (26). For the gel retardation assay, 1 μl of mouse NIH 3T3 nuclear extracts (3–5 μg of protein) or 2–3 fmol of c-Krox or truncated c-Krox proteins (unless otherwise specified) were incubated for 15 min at room temperature with 5 fmol of end-labeled oligonucleotides in a 10-μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 1 mM dithiotreitol, 100 mM NaCl, 0.05 mg/ml bovine serum albumin, 0.1% Nonidet P-40, 10% glycerol, 25 μM ZnCl2, and 4 μg of poly(dI-dC)poly(dI-dC) that was omitted when using recombinant proteins. Electrophoresis conditions were as described previously (15). The oligonucleotides used in this study are presented in Table I. For DNAase I footprint assays, plasmid pG60 was digested with EcoRI (222), dephosphorylated, end-labeled at the EcoRI site using T4 polynucleotide kinase and [γ-32P]ATP, and then cut by HindIII. The 338-bp DNA fragment carrying the mouse α1(I)
collagen promoter was isolated as described (13). Plasmid pA21003 was digested at the BglII site (−350), dephosphorylated, and end-labeled as above, and then cut by HindIII to generate a 402-bp a2(I) collagen promoter DNA fragment. The DNA binding reactions were performed as described previously (13). The gel retardation templates were subjected to the A + G Maxam-Gilbert sequencing reaction (29) and loaded on the same gel to identify the protected sequences. After electrophoresis, the gel was subjected to autoradiography at −80°C.

RNA Analysis and In Situ Hybridization—NIH3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS). At 80% confluency, the cells were starved for 72 h in Dulbecco's modified Eagle's medium containing 1% FCS. The medium was then changed to Dulbecco's modified Eagle's medium containing 20% FCS. The cells were harvested at various times after serum addition. Poly(A)+ RNA was isolated with a Fast track kit (Invitrogen, San Diego, CA) using the manufacturer's instructions. Analysis of mRNA expression was performed by Northern blot as described previously (30). Briefly, poly(A)+ RNA was fractionated by electrophoresis and transferred to Hybond-N+ (Amersham Corp.). The probe used included 513 bp of c-Krox coding sequence 5′ of the zinc finger domain. Prehybridization and hybridization were carried out at 65°C in rapid hybridization buffer (Amersham Corp.). Final washes were in 0.1 x sodium chloride/sodium citrate and 0.1% SDS at 65°C for 15 min. RNase protection assays were performed using a RPA II kit (Ambion Inc., Austin, TX) using the manufacturer's instructions. Plasmid pc-Krox1 (15) was used to transcribe a 160-nucleotide antisense RNA probe labeled with [32P]CTP. The β-actin probe provided in the kit was used as recommended by the manufacturer. The reaction products were run on a denaturing 6% polyacrylamide gel and subjected to autoradiography.

Whole-mount in situ hybridization was performed as described previously (31) using a labeled riboprobe corresponding to 513 bp of c-Krox sequence 5′ of the zinc finger coding sequences. Section in situ hybridization procedures were as described (32) with the following modifications. Sections of 8 µm were mounted onto polylysine-treated slides. The hybridization and posthybridization washes were performed as described (33). Hybridization was carried out overnight at 60°C, and the stringency washes were at 62°C. Exposure times were 4–16 days. Autoradiography and photography were performed as described (32).

RESULTS

c-Krox Binds to the B Element of the Mouse a2(I) Collagen Promoter—We showed earlier that an element analogous to the B element of the a1(I) gene promoter is present in the mouse a2(I) collagen gene promoter (14). Since this element, called B a2(I), binds the same factor present in NIH3T3 nuclear extracts as the one binding to the A a1(I) and B a1(I) elements, we asked whether recombinant c-Krox could bind to the B a2(I) element in GRA. The purified His-tagged c-Krox protein bound to the labeled wild-type B a2(I) oligonucleotide but not to the labeled mutant B a2(I) oligonucleotide (Fig. 1, lanes 1 and 3). As previously reported, His-tagged c-Krox binds as a dimer (15). The protein-DNA complex formed upon incubation of NIH3T3 nuclear extracts with the labeled B a2(I) oligonucleotide migrated with a mobility nearly identical to that of the c-Krox-DNA complex (Fig. 1, compare lanes 2 and 3), suggesting that the factor present in NIH3T3 nuclear extracts and binding to this probe is related to c-Krox.

In DNA competition experiments, the binding of His-tagged c-Krox to the labeled B a2(I) oligonucleotide was inhibited by excess amounts of either the wild-type A a1(I) oligonucleotide or the wild-type B a2(I) oligonucleotide but not by the same molar amount of either the mutant A a1(I) oligonucleotide or the mutant B a2(I) oligonucleotide containing a 3-bp substitution mutation that abolishes c-Krox binding (13, 15) (Fig. 1, lanes 4–16). As further proof of the specificity of c-Krox binding to B a2(I) oligonucleotide, we performed DNA competition experiments, using oligonucleotides containing either an NF1 binding site (37) or a binding site for AP2, another transcription factor binding to a GC-rich sequence (27). Excess molar amounts of neither of these two oligonucleotides abolished the binding of His-tagged c-Krox to the labeled B a2(I) oligonucleotide (Fig. 1, lanes 18–23). Thus, the direct binding and the DNA competition experiments indicate that c-Krox binds specifically to the B a2(I) site present in the promoter of the mouse a2(I) collagen gene.

c-Krox Binds to Several Sites in the Mouse a1(I) and a2(I) Collagen Gene Promoters—Several GC-rich sequences, some of...
them playing a crucial role in the basal activity of this promoter or in its regulation by various cytokines (10, 11), are present in the promoter of the α2(I) collagen gene. Thus, we asked whether c-Krox could bind to other GC-rich sequences in this promoter. We initially performed a DNase I footprint assay using as a template the −350 to +54 fragment of the α2(I) collagen promoter. As shown in Fig. 2A, c-Krox binds to three sites in this promoter fragment. The first protected region, located between −264 and −277, is centered by a GC-rich sequence identical to other c-Krox binding sites. The second protected region, located between −112 and −189, contains the B α2(I) element between −175 and −143. Surprisingly, the third and most proximal c-Krox binding site, located between −72 and −109, includes the CCAAT box that can also bind a multimeric activator of transcription called CBF (12, 38, 39). The presence of a binding site for c-Krox near the CCAAT box of α2(I) promoter prompted us to determine whether c-Krox binds to a site adjacent to the CCAAT box in the α1(I) promoter. When we used the −220 to +120 α1(I) collagen promoter fragment as a template in DNase I footprint assay we also observed that, besides its known binding sites, c-Krox binds, although less well, to another site located between −65 and −94 and containing the proximal CCAAT box (Fig. 2B).

To test the specificity of c-Krox binding to these two additional sites in the α2(I) collagen promoter, we performed GRA using as a probe either an oligonucleotide containing the −264 to −277 sequence or an oligonucleotide centered by the CCAAT box of the mouse α2(I) collagen promoter. His-tagged c-Krox binds to the labeled −264 to −277 α2(I) oligonucleotide. This binding was specific since it could be inhibited by a molar excess of the wild-type B α2(I) oligonucleotide but not by its mutant counterpart, by a molar excess of the wild-type −264 to −277 oligonucleotide, but not by a mutant −264 to −277 oligonucleotide containing a mutation that abolishes c-Krox binding to DNA, by a molar excess of the wild-type CCAAT α2(I) oligonucleotide, but not by its mutant counterpart (Fig. 3A, lanes 1–20). When we performed the reverse experiments using the wild-type B α2(I) oligonucleotide as a probe, we observed that c-Krox binding to this oligonucleotide was competed away by a molar excess of the wild-type −264 to −277 oligonucleotide but not by its mutant counterpart (Fig. 3B).

His-tagged c-Krox did also bind to the labeled CCAAT α2(I) oligonucleotide. This binding was competed away by the wild-type B α2(I) oligonucleotide but not to the same extent by its mutant counterpart; c-Krox binding to the CCAAT α2(I) oligonucleotide was also inhibited by the wild-type CCAAT α2(I) oligonucleotide but not to the same extent by a mutant CCAAT α2(I) oligonucleotide containing a 3-bp mutation just 5' of the CCAAT pentanucleotide (Fig. 3C, lanes 8–12), indicating that there was a true c-Krox binding site in the vicinity of the CCAAT box in the α2(I) collagen promoter (Fig. 3C, lanes 1–12). Inspection of the sequence of the promoter showed a c-Krox...
consensus binding site immediately 5' of the CCAAT pentanucleotide. Taken together these results indicate that, under the conditions of GRA, c-Krox could bind specifically to three sites in the minimal promoter region of the mouse α2(I) collagen promoter, all containing the same consensus sequence (see Table I). One site is located between −264 and −277; the second site is the B α2(I) element located between −175 and −143, and the third binding site of lower affinity is located immediately upstream of the CCAAT box.

The Transactivation Domain of c-Krox Is Included in the Zinc Finger Domain and the C-terminal Domain—We had shown earlier that c-Krox acts as an activator of transcription in a DNA cotransfection assay (15). To identify the location of potential transactivating sequences in c-Krox, we used the GAL4 transcription factor of the yeast Saccharomyces cerevisiae. GAL4 contains two separable domains, the DNA-binding domain and the activation domain (40). The GAL4 DNA-binding domain, which is contained in the 147 N-terminal amino acids, can bind to a specific sequence called the upstream activator site-dependent CAT gene expression. A construct containing the full-length c-Krox had little but reproducible ability to stimulate the promoter activity of the reporter gene in six different experiments. Constructs containing the N-terminal part of c-Krox and the zinc finger domain did not activate transcription. However, a construct containing the zinc finger and the C-terminal domains increased the expression of the reporter gene 5-fold above basal levels in six independent experiments. Constructions containing either the zinc finger domain alone or the C-terminal domain alone resulted in no activation of transcription (Fig. 4).

To verify whether the chimeric proteins encoded by the different constructs shown in Fig. 4 were expressed, we performed Western blotting using an anti-GAL4 antibody. Fig. 5 shows that cells transfected with plasmids coding for GAL4 DNA-binding domain, GAL4-c-Krox, GAL4-Δc-Krox (Zn + C), GAL4-Δc-Krox (Zn), GAL4-Δc-Krox (N), GAL4-Δc-Krox (N + Zn), all expressed the proper recombinant proteins. Thus, the results of Fig. 4 confirm our earlier data showing that c-Krox acts as an activator of transcription; they also show that the zinc finger domain and the C-terminal domain of c-Krox are both required for transactivation.

A Homodimerization Domain Is Present at the C-terminal End of c-Krox—It was shown earlier that Krüppel can homodimerize through sequences located within the C terminus (41). To test the ability of c-Krox to form homodimers, we used His-tagged c-Krox and different deletion mutants of His-tagged c-Krox and the wild-type A α2(I) oligonucleotide in GRA. Similar results were obtained when using the B α2(I) oligonucleotide as a probe (data not shown). When we used the full-length c-Krox protein in this assay, we could see a protein-DNA complex formed as a doublet (Fig. 6, left panel, lanes 1–6). This doublet was observed whether we used low or high concentrations of c-Krox. However, at high concentrations of c-Krox, a third complex of slower mobility also appeared, indicating that c-Krox can homodimerize (Fig. 6, left panel, lanes 4–6). To delimit the region required for c-Krox homodimerization, we used two c-Krox deletion mutants. Δc-Krox (Zn + C) contains the zinc finger and the C-terminal domains of c-Krox and Δc-Krox (Zn) contains only the zinc finger domain. When using increasing amounts of Δc-Krox (Zn + C), we observed the appearance of a third complex of slower mobility (Fig. 6, middle panel, lanes 1–6) and the disappearance of the protein-DNA complex of faster mobility (Fig. 6 middle panel, lanes 5, 6); this indicates that this c-Krox deletion mutant can also bind as a homodimer. Interestingly, the protein-DNA complex of slower mobility could be detected with less recombinant protein than what was required to observe it when using full-length c-Krox. In contrast, Δc-Krox (Zn) formed only a single complex when incubated with DNA, regardless of the amount of recombinant protein used (Fig. 6, right panel, lanes 1–6). These results indicate that c-Krox can homodimerize, this function requiring sequences present at the C terminus of the molecule. The fact that Δc-Krox (Zn + C) homodimerizes more readily than wild-type c-Krox suggests that the N terminus of c-Krox could play an inhibitory role in this process.

c-Krox Is an Early Response Gene—c-Krox belongs to a family of genes whose expression is up-regulated by serum in cultured cells (21, 42). To test if c-Krox was up-regulated in these conditions, we prepared RNA from NIH3T3 cells maintained in quiescent state (G0) by serum deprivation for 48 h and exposed these cells to FCS for different lengths of time, so they would reenter G1 phase and proliferate (43, 44). These RNA were analyzed by Northern blot using a probe corresponding to the 5’ end of c-Krox cDNA to avoid cross-hybridization with trans-

### Table II

| Comparison of various binding sites for c-Krox | The identical basepairs among the binding sites are indicated in bold. |
|---------------------------------------------|---------------------------------------------------------------|
| A α2(I) | 5'−TGGGAGGAGGAGG−3' |
| B α2(I) | 5'−TGGGAGGAGGAGG−3' |
| −264 α2(I) | 5'−TGGGAGGAGGAGG−3' |
| CCAAT α2(I) | 5'−AATGGGAGGAGG−3' |
| Consensus sequence | 5'−GAGGAGGAGGAGG−3' |

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**Fig. 4. Transactivation by GAL4-c-Krox fusion proteins.** NIH3T3 cells were transiently transfected with 5 μg of the indicated expression vector and reporter plasmid containing four copies of the GAL4 recognition site. Transcriptional activity is expressed as CAT activity relative to control. A typical experiment is presented. The details of the constructs are described under “Materials and Methods.” GAL4 BD, GAL4 DNA binding domain; aa, amino acid(s).
scripts of other zinc finger proteins. As shown in Fig. 7, we detected only a low level of expression of c-Krox in quiescent cells, but addition of serum led to a rapid increase of c-Krox expression (10-fold) after 60 min when it reached a maximum and began to decrease after 180 min (data not shown). Addition of cycloheximide to the cells did not prevent the increase in c-Krox expression induced by FCS, indicating that de novo protein synthesis was not required for c-Krox activation. Strong superinduction of c-Krox expression by cycloheximide, a characteristic of several immediate early response genes (45), was not observed. Subsequent rehybridization of the filter with a probe corresponding to glyceraldehyde-3-phosphate dehydrogenase, whose expression does not vary during G0/G1 transition was investigated by Northern blot analysis of poly(A)+ RNA extracted from NIH3T3 fibroblasts. The numbers above each lane indicate time points (min). Zero corresponds to no stimulation by serum: CHX, cycloheximide.

c-Krox in skin in postnatal life and its absence in bone (15). We then asked whether c-Krox and type I collagen mRNAs colocalized during development. We performed section in situ hybridization on 14.5 dpc mouse embryos, a time when type I collagen expression is readily detectable. As shown in Fig. 8c, c-Krox and type I collagen mRNAs colocalized in skin, whereas there was no expression of c-Krox in bone.

c-Krox Mapped to the X Chromosome—In situ hybridization experiments were carried out using metaphase spreads from a female mouse, in which all the autosomes except 19 were in the form of metacentric robertsonian translocations. Concanavalin A-stimulated lymphocytes were cultured at 37 °C for 72 h, with 5-bromodeoxyuridine added for the final 6 h of culture (60 μg/ml of medium), to ensure a chromosomal R-banding of good quality. In the 150 metaphase cells examined after in situ hybridization, there were 168 silver grains associated with chromosomes, and 49 of these (29.1%) were located on chromosome X. The distribution of grains on this chromosome was not random; 37 out of 49 (75.5%) of them mapped to the (A1-A2) region of chromosome X with a maximum in the A2 band (Fig. 9). These results allow us to map c-Krox in the XA1-XA2 bands of the murine genome. This is the first Krox gene located on the X chromosome.

**DISCUSSION**

In this paper we extended our characterization of c-Krox by defining several binding sites for this protein in the promoter elements of the mouse α1(I) and α2(I) collagen genes, by performing structure/function analysis, and by initiating the study of c-Krox pattern of expression during development.

c-Krox was cloned as a cDNA encoding a transcription factor binding to two identical GC-rich sequences present in the promoter of the mouse α1(I) collagen gene (15). Our present study shows that c-Krox binds not only to its cognate sequence in the
α2(I) collagen promoter (14) but also to two other sites in this promoter and an additional one in the promoter of the α1(I) collagen gene. Interestingly, one of these sites overlaps the binding site of CBF, a heteromeric activator of transcription that binds to the CCAAT box (12, 37, 46). The fact that c-Krox and CBF bind to overlapping sequences suggests that the binding of c-Krox to DNA may prevent, at least partially, binding of CBF to DNA. If this hypothesis is correct it would explain why the GC-rich sequences where c-Krox binds in the promoter of both type I collagen genes were initially thought, based on DNA transfection experiments, to act as inhibitors of transcription.

Indeed, by introducing mutations in these GC-rich sequences that abolish binding of nuclear factors to them we may have increased the binding of CBF to the CCAAT box and therefore increased the activity of these promoters (13, 14). Further analysis will be needed to analyze possible reciprocal interactions between c-Krox and CBF at this site.

The analysis of the sequence of the different c-Krox binding sites allowed us to define a core consensus sequence 5'-GGGAGGG-3'. Using this core sequence we searched for potential c-Krox target genes, and found that the common promoter shared by α1(IV) and α2(IV) collagen genes contains a perfect
c-Krox binding site. DNA binding studies have shown that c-Krox could bind to this site. Although there is no direct evidence yet that c-Krox modulates the expression of these genes, this possibility is worth investigation. Likewise, we are currently testing whether c-Krox could regulate expression of other genes coding extracellular matrix proteins.

The structure/function analysis of c-Krox showed several features characteristic of the Krüppel and Krox proteins (41, 47, 48). To define c-Krox activation domain, we fused distinct domains of c-Krox to the DNA-binding domain of the yeast transcription factor GAL4 and used them in DNA cotransfection assay with a reporter vector containing four GAL4 DNA target sequences. By this assay we defined the zinc finger domain and the C-terminal part of the protein as the transcription activation domain. The C-terminal domain of Krüppel and YY1, a human homologue of Krüppel, have been also shown to contribute to the transcriptional activity of these two proteins (41). Unlike the case for YY1 and Krüppel, we did not detect any repression of transcription activity by any domain of c-Krox even when we used different concentrations of the expression vector (data not shown). The low level of transcriptional activity of c-Krox we report here has also been observed with other Krox genes such as Krox 24 and more recently YY1 when tested in tissue culture experiments (42, 45). Like SF-1, another zinc-finger-containing transcription factor (49), the N-terminal domain of c-Krox does not have any transcriptional activity and may modulate the function of the zinc finger and C-terminal domains.

c-Krox is able to homodimerize, and the homodimerization domain is located in the C-terminal domain. The dimerization ability of c-Krox, the similarity between c-Krox binding site and Sp1 binding site, and the close vicinity of a bona fide Sp1 binding site raise the hypothesis that c-Krox and Sp1 could compete for the same recognition site. Although there is no direct evidence yet that c-Krox could bind to this site, we currently testing whether c-Krox could regulate expression of other genes coding extracellular matrix proteins.

Finally we show that c-Krox expression is detectable as early as 9.5 dpc. When we performed whole-mount in situ hybridization we did not observe any c-Krox expression at 9.5 dpc. This may be due to the low sensitivity of this assay for lower abundance messages compared with other methods. In contrast, in three different embryo preparations we observed expression of c-Krox mRNA above background at 10.5 dpc only in the dermatomes, which are somatic derivatives from which dorsal dermis will be generated. Furthermore, c-Krox and type I collagen mRNAs colocalized in developing bone but not in developing skin. This observation is consistent with our finding that c-Krox is more highly expressed in skin after birth and is in agreement with several reports showing that different cis-acting elements control type I collagen gene expression in skin and bone (51–53). This result does not exclude that c-Krox is expressed in other structures during development. Nevertheless, its predominant expression in skin during development and postnatal life suggests that c-Krox may participate in the differentiation of this tissue and is an incentive to study c-Krox function in vivo.

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43. Pardee, A. B., Dubrow, R., Hamlin, J. L., and Kletzien, R. F. (1978) Annu. Rev. Biochem. 47, 715–750
44. Chavrier, P., Zerial, M., Lemaire, P., Almendral, J., Bravo, R., and Charnay, P. (1988) EMBO J. 7, 29–35
45. Lau, L. F., and Nathans, D. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1182–1186
46. Maity, S. N., Sinha, S., Ruteshouser, E. C., and de Crombrugghe, B. (1992) J. Biol. Chem. 267, 16574–16580
47. Gashler, A. L., Swaminathan, S., and Sukhatme, V. P. (1993) Mol. Cell. Biol. 13, 4556–4571
48. Buchmeyer, S., Park, K., and Atchison, M. L. (1995) J. Biol. Chem. 270, 30213–30220
49. Shen, W.-H., Moore, C. D., Ikeda, Y., Parker, K. L., and Ingraham, H. A. (1994) Cell 77, 651–661
50. Seto, E., Lewis, B., and Shenk, T. (1993) Nature 365, 462–464
51. Liska, D. A. J., Reed, M. J., Sage, E. H., and Bornstein, P. (1994) J. Cell. Biol. 125, 695–704
52. Bogdanovic, Z., Bedalov, A., Krebsbach, P., Pavlin, D., Woody, C. O., Clark, S. H., Thomas, H. F., Rowe, D. W., Kream, B. E., and Lichtler, A. C. (1994) J. Bone Min. Res. 9, 285–291
53. Rossert, J., Eberspaecher, H., and de Crombrugghe, B. (1995) J. Cell. Biol. 129, 1421–1432
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