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Biochemistry

c-Krox, a transcriptional regulator of type I collagen gene expression, is preferentially expressed in skin

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ABSTRACT **We have cloned a mouse cDNA that is a member of the Krox gene family and encodes a protein we have named c-Krox. The c-Krox protein contains three zinc fingers of the Cys_2His_2 type. c-Krox binds specifically to a guanine-rich cis-acting element present twice in the promoter element of the mouse α1(I) collagen gene. Study of c-Krox gene expression shows that c-Krox is markedly enriched in skin, one of the two major sites of type I collagen synthesis, but is absent in bone, the other main type I collagen-producing tissue, indicating that type I collagen gene expression is differentially regulated in skin and bone. DNA transfection experiments in mouse NIH 3T3 fibroblasts, cells that express the c-Krox gene, or in *Drosophila* S2 cells, which do not express c-Krox, reveal that c-Krox can activate transcription of a reporter gene linked to several copies of its binding site in the α1(I) collagen promoter. Thus, c-Krox is only the second member of the Krox family for which a target gene has been identified. The selective spatial pattern of expression of its mRNA and its transcription activation ability suggest that c-Krox may be an important regulator of type I collagen skin specific expression in physiologic conditions and in fibrotic diseases such as scleroderma.**

Type I collagen is a major constituent of the extracellular matrix of bone and skin. Its synthesis is restricted to two cell types of mesenchymal origin, the osteoblasts and fibroblasts (1). Type I collagen is a heterotrimer consisting of two α1(I) chains and one α2(I) chain that are encoded by two different genes (1). Changes in the synthesis of type I collagen occur during embryonic development and wound healing and in many pathological situations such as liver, lung, and skin fibrosis and scleroderma (2, 3). In most of these situations the increase in synthesis of the type I collagen chains is mediated by transcriptional mechanisms (1).

An analysis of the cis-acting elements of the mouse α1(I) collagen gene was initiated with the long-term objective of understanding the molecular mechanisms controlling type I collagen gene expression in normal and pathological conditions. Previous studies have shown that 220 bp of 5′ flanking sequence of the mouse α1(I) collagen gene acts as a strong promoter when tested in an *in vitro* transcription assay (4) or in DNA transfection experiments (5). Four different cis-acting elements that bind three distinct trans-acting factors were further identified (5).

One of these cis-acting elements, whose center contains a G-rich sequence, is present twice in the α1(I) promoter, where it is called Aα1(I) and Bα1(I) (5). These two G-rich elements specifically bind a factor present in nuclear extracts of NIH 3T3 fibroblasts (5). Site-specific mutagenesis of this element increases the activity of the α1(I) collagen promoter in DNA transfection experiments, suggesting that this element acts as an inhibitor of α1(I) collagen gene transcription (5). This G-rich sequence is present in the promoter of the LpS1 gene of sea urchin, where it acts as an activator of transcription (6), indicating that the factor(s) binding to this sequence could act as either activators or inhibitors of transcription, depending on the promoter context.

In this paper we present the characterization of a zinc finger protein encoded by a gene belonging to the mouse Krox gene family (7). This protein, termed c-Krox, binds specifically to the G-rich elements Aα1(I) and Bα1(I) and regulates expression of an α1(I) collagen promoter-luciferase chimeric gene through the Aα1(I) site. Interestingly, c-Krox mRNA is preferentially expressed in dermis postnatally but is absent in bone. Together, our results suggest that c-Krox plays an important role in the skin-specific expression of type I collagen genes and possibly of other genes expressed in dermis.†

MATERIALS AND METHODS

Library Screening. The c-Krox clone was isolated by oligonucleotide screening of an oligo(dT)-primed λgt11 cDNA expression library generated from mouse NIH 3T3 fibroblasts (8). A double-stranded oligonucleotide containing a multimerized Aα1(I) binding site was used to probe the library.

5′-GATCCTTGCGGGAGGGGGGCGCGCTGGGTGGACA-3′
3′-GAACGCCCTCCCCCCGCGCGACCCACCTGACTAG-5′

One positive clone from the primary screening called MM13 was plaque purified, and its cDNA insert was subcloned into the pBluescript KS vector (Stratagene) for sequence analysis. The 5′ end of the MM13 clone was used to screen additional libraries to obtain overlapping and longer clones. The various cDNA clones selected from these libraries were sequenced with the Sequenase version 2 sequencing kit (United States Biochemical).

Purification of c-Krox Recombinant Protein and Gel Retardation Assay. Plasmid pV2b was used to create a histidine-tagged c-Krox fusion protein (9). A *Nae* I–*Eco*RI insert from the c-Krox cDNA clone was ligated in frame with coding sequence for the histidine residues in the pV2b vector. This fusion protein, containing aa 2–427 of c-Krox, was purified to homogeneity on Ni^{2+}-bound iminodiacetic acid agarose resin (10). Gel retardation assays were performed as described (5) except that the glycerol concentration was lowered to 5% in the binding buffer. The sequences of Aα1(I) and Bα1(I) double-stranded oligonucleotides, wild type or mutant, were identical to those previously described (5).

RNA Analysis. Isolation of total cellular RNA from various mouse tissues and Northern blot analysis were performed as described (11). Poly(A)$^+$ RNA from NIH 3T3 fibroblasts was isolated with a Fast Track kit (Invitrogen). Analysis of mRNA expression by RNase protection assay was performed with an Ambion RPA II kit (Ambion, Austin, TX). Plasmid pc-Krox1 was constructed by inserting a 117-bp *Spe* I–*Rsa* I

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*To whom reprint requests should be addressed.
†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L35307).

A

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   1  AATTCCGGGCTGTGTATCCGAATATTTATTTTGATTTTTTAAATAAATTGAACTATATTT
  61  CATAAGCCCTGAGATATTAGATAACCTGCTAATGATTGTTCACTTTTAATGTATATTCTA
 121  CCCCATTTGTCCTAACTCTACATAAGTATTTTTAAATTAGTCACACACGTTGAAACTAAT
 181  TCAATAAAATTTATCATTGTATAGCTAAGACTACTAATAAGGCCTTTCAGTAAGAGTTGC
 241  ATATTTGATGTATTACTTTCTGGGAAGCAACCCTTAAAAACTGTATCTATAATTCTGTCA
 301  CCAATTAGCGCTCTCCTTCTGGTTAATTTTCCACGGAGGACCAGACTCTTTTCCTTTCTT
 361  TCTTTTATTCTCCCTCAAACATAAACACTGGTTTTCCAACTGAATAACTGCCAAAACCAA
 421  CCTGCCAGGTGAACGCATGTGAATGCCACGTGAACACCACGTTAAGAGCCTGTCTGCCTG
 481  GAGAGAAACATGCAGAGCACACATCCGATAGGTACCTGCCCATTTCTCATGGAAGCAATG
 541  GATTGTAAGGCAGTTGTCTGAGTCATATACTGTTGATCATATACTTTACTAATTTTGTTC
 601  ACCCAGGAAATCTGGTCGATCATATTTAATTCCACCTCAAGTGTAAATGTAAGAAACTGG
 661  CATTAAAACAAAAAAAAAAAAATCAATGGATTTGCCAGTTCTCAGGAAAAGAAAACTAGT
 721  TAGATGGGGCGGGTGTATGTGAGCTGGACTTTGTAGGCAGAGGCCCTAGGTGCCCTGCTC
 781  GAGTTTGCTTACACAGCCACACTGACCACCAGCAGCGCCAATATGCCGGCTGTACTCCAA
                                                 M  P  A  V  L  Q     6
 841  GCTGCTCGGCTACTGGAAATCCCGTGTGTCATCGCTGCTTGCATGGAGATTCTACAAGGC
       A  A  R  L  L  E  I  P  C  V  I  A  A  C  M  E  I  L  Q  G    26
 901  AGTGGGCTGGAAGCCCCCAGCCCCGATGAGGATGACTGTGAGCGAGCCCGACAGTACCTG
       S  G  L  E  A  P  S  P  D  E  D  D  C  E  R  A  R  Q  Y  L    46
 961  GAGGCTTTTGCCACTGCCACCACCACAGCCTCAACCTCAGGAATGCCCAACGGTGAAGAC
       E  A  F  A  T  A  T  T  T  A  S  T  S  G  M  P  N  G  E  D    66
1021  AGCCCTCCACAGGTGCCCCTCCTACCGCCGCCGCCACCGCCACCTCGACCTGTTGCCCGT
       S  P  P  Q  V  P  L  L  P  P  P  P  P  P  P  R  P  V  A  R    86
1081  CGCAGCCGCAAACCCCGGAAAGCTTTTCTTCAAACCAAAGGGGCCCGAGCAAACCATCTG
       R  S  R  K  P  R  K  A  F  L  Q  T  K  G  A  R  A  N  H  L   106
1141  GTCCCTGAGGCACCCACAGTACTCACCCATCCCTTGACCTACGAAGAAGAAGAGATGGTT
       V  P  E  A  P  T  V  L  T  H  P  L  T  Y  E  E  E  E  M  V   126
1201  GGTAGATTGGGTAACAGTGGGGGCAGTGGGCTCGGAGATAGCTATAGTCCTCCTACAGGT
       G  R  L  G  N  S  G  G  S  G  L  G  D  S  Y  S  P  P  T  G   146
1261  GCCGCCTCACCTACCGAGGGGCCCCTGAACTATGAAGTCTTTGAAGGTGAGGAAGAAGAG
       A  A  S  P  T  E  G  P  L  N  Y  E  V  F  E  G  E  E  E  E   166
1321  GAGGAGATGGCATACCCCCCAGGCTACGGGCTAGCCCAGAGTAATGAGCCCTCGCTATCA
       E  E  M  A  Y  P  P  G  Y  G  L  A  Q  S  N  E  P  S  L  S   186
1381  CCAGAGGAGCTGGGCTCAGATGAAGATCCCATCGACCCCGACCTGATGGCTTACCTAAGT
       P  E  E  L  G  S  D  E  D  P  I  D  P  D  L  M  A  Y  L  S   206
1441  TCGCTGCACCAGGGACGCCCTGACACCAGGCCTGGATGGCAAGACAAGCTGGTGCGTAAA
       S  L  H  Q  G  R  P  D  T  R  P  G  W  Q  D  K  L  V  R  K   226
1501  CGCCGTTCACAGATGCCCCAAGAGTGCCCAGTCTGTCACAAGATAATCCACGGGGCAGGC
       R  R  S  Q  M  P  Q  E  C  P  V  C  H  K  I  I  H  G  A  G   246
1561  AAACTGCCTCGGCACATGAGGACCCACACTGGTGAGAAGCCCTTTGCCTGTGAGGTCTGC
       K  L  P  R  H  M  R  T  H  T  G  E  K  P  F  A  C  E  V  C   266
1621  GGCGTCCGATTCACCAGGAATGACAAGCTGAAGATCCACATGCGGAAGCACACAGGAGAA
       G  V  R  F  T  R  N  D  K  L  K  I  H  M  R  K  H  T  G  E   286
1681  CGCCCCTACTCGTGCCCCCACTGCCCAGCCCGCTTCCTGCATAGCTACGACCTCAAGAAC
       R  P  Y  S  C  P  H  C  P  A  R  F  L  H  S  Y  D  L  K  N   306
1741  CACATGCACCTGCACACCGGGGACCGGCCCTATGAGTGCCACCTGTGCCACAAGGCCTTC
       H  M  H  L  H  T  G  D  R  P  Y  E  C  H  L  C  H  K  A  F   326
1801  GCCAAGGAGGACCACCTGCAGCGCCATCTCAAGGGTCAGAACTGCCTGGAGGTGCGCACC
       A  K  E  D  H  L  Q  R  H  L  K  G  Q  N  C  L  E  V  R  T   346
1861  CGAAGGCGCCGCAAGGATGACGTGGCAGCCCCTCACTACCCACCGCCCTCGACCACCACC
       R  R  R  R  K  D  D  V  A  A  P  H  Y  P  P  P  S  T  T  T   366
1921  TCATCCCCTGCTGGCCTCGACCTTTCCAATGGCCACCTGGACACCTTCCACCTCTCTCTG
       S  S  P  A  G  L  D  L  S  N  G  H  L  D  T  F  H  L  S  L   386
1981  GCTCGATTCTGGGAGCAGTCGGCCACCACGGGACCCCCAGTCACTACACAAGGGCCCCCT
       A  R  F  W  E  Q  S  A  T  T  G  P  P  V  T  T  Q  G  P  P   406
2041  GAGGAGGAGGAAGAGGAAGGGACACCCACCACACCACAGGCCGAAGGTGCCATGGAGTCC
       E  E  E  E  E  E  G  T  P  T  T  P  Q  A  E  G  A  M  E  S   426
2101  TCTTAAAAGAGGGATGAATGGGCAGGCTGGAACAGCCTGGGACACCACGCCAAGCAACGG
       S                                                            427
2161  GGAGCACATAAAACAAGTGGTATCGGGGTCGGGGGTGCTGAACCTTGTTGGTATCAGAAT
2221  CGAACGCCTCTTCCCCCCAGAGCCTTCATTCCAATTACAAGCTGAGGTTTTGGGGCACAA
2281  GCTCTCTAGATTGGGGTGATCACCCAAGGAGTTAAACATGAAATATATGTCTGTTTGTCT
2341  GTCAAAAAAAAAA
```

B

Sequence	Protein	Identity, %	Similarity, %
CPVCHKIIHGAGKLPRHMRTHTGEKPFACEVCGVRFTRNDKLKIHMRKHTGERPYSCPHCPARFLHSYDLKNHMHLH	C-KROX		
--E-D-R-TRDHH-KT---W-------H-SH--RQSVQVAN--R-W-V-------T-EI-D---SD-N---S--L--	KRUPPEL	45	56
--AEG-D-R-SRS-E-T----I----H---Q-R--MRN-S-S-H-TT---T-------A-DY-GR--A--D-R-R-TK--	KROX20	42	56
---ES-D-R-SRS-E-T----I-------Q-R--MRN-S-S-H-TT---T-------A-DI-GR--A--D-R-R-TK--	KROX24	44	59
-DE-G-T--QS-S-L--Q-I-------T-----K--IERSS-T--Q-T-------K-HE-GKA-S--M--TV--RT-	KROX2	41	59

FIG. 1. Sequence analysis of c-Krox. (*A*) Nucleotide sequence and deduced amino acid sequence of c-Krox cDNA. The ATG initiation codon and the TAG termination codon are underlined, as are the three zinc fingers. Numbers at left refer to nucleotide position, and those at right refer to amino acid position. (*B*) Homologies between the c-Krox zinc finger domain and the zinc finger domains of other proteins. Dashes represent amino acids identical to c-Krox. The cysteines and histidines of the zinc fingers are in boldface type.

fragment from c-Krox cDNA into the *Spe* I/*Hin*cII sites of pBluescript. pc-Krox1 was linearized by *Xba* I and T3 RNA polymerase was used to transcribe a 160-nt antisense RNA probe labeled with [α-^{32}P]CTP.

Cell Lines and DNA Transfections. DNA was transfected into mouse NIH 3T3 cells by calcium phosphate precipitation (12). *Drosophila* S2 cells were transfected by electroporation. In reporter plasmids pK1A4Luc and pK1A4mLuc, four tandem copies of either the wild-type or the mutant Aα(I) binding site are present upstream of the 86-bp α1(I) basal promoter fused to the luciferase gene (5). The c-Krox expression vector was created by insertion of a full-length c-Krox cDNA into the *Eco*RI site of pEMSV vector, which contains the Moloney sarcoma virus long terminal repeat and the simian virus 40 polyadenylylation signal. For DNA transfection experiments in *Drosophila* S2 cells, c-Krox cDNA

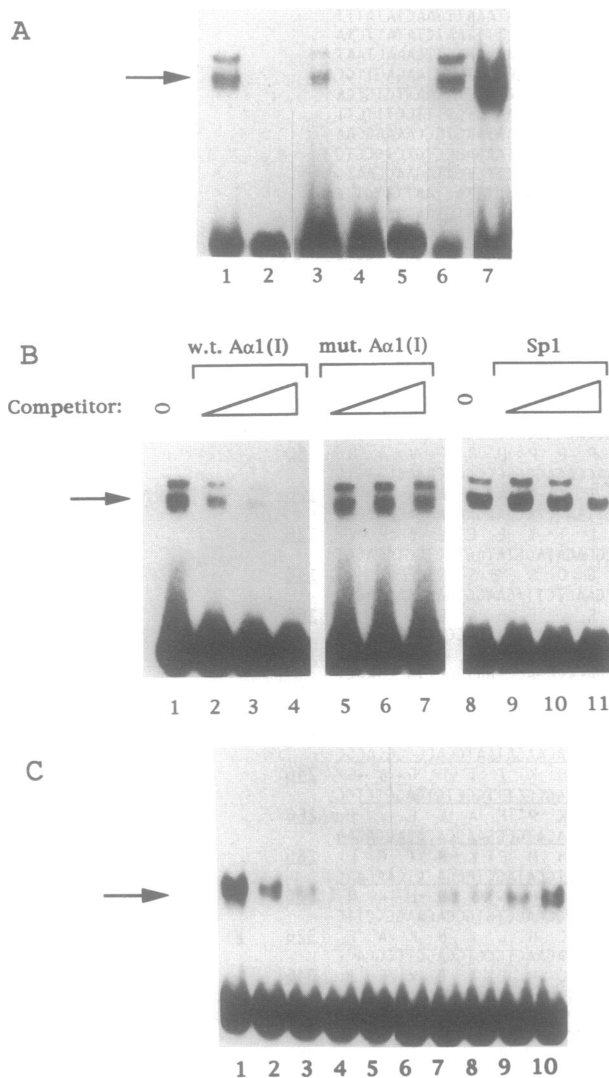

FIG. 2. DNA-binding assays. (*A*) DNA binding was analyzed by gel mobility-shift assay. ^{32}P-labeled Aα1(I) (lanes 1, 6, and 7) or Bα1(I) (lane 3) double-stranded oligonucleotides, or their mutant counterparts (lanes 2 and 4, respectively), or an Sp1 consensus double-stranded oligonucleotide (lane 5) were incubated for 15 min with 2 fmol of recombinant c-Krox protein (lanes 1–6) or 1 μl of NIH 3T3 nuclear extract (5 μg of protein) (lane 7). c-Krox protein–DNA complex is indicated by an arrow. (*B*) A labeled Aα1(I) double-stranded oligonucleotide was incubated with recombinant c-Krox protein in competition experiments with 20-, 50-, or 75-fold molar excess of either wild-type Aα1(I) oligonucleotide (lanes 2–4), a mutant Aα1(I) oligonucleotide (lanes 5–7), or an oligonucleotide containing an Sp1 binding site (lanes 9–11). (*C*) A labeled Aα1(I) double-stranded oligonucleotide was incubated with NIH 3T3 nuclear extract in the presence of no EDTA (lane 1), 0.5 mM EDTA (lane 2), 1 mM EDTA (lane 3), 2 mM EDTA (lane 4), 3 mM EDTA (lane 5), or 2 mM EDTA plus 0, 50, 100, 300, or 400 μM $ZnCl_2$ (lanes 6–10, respectively).

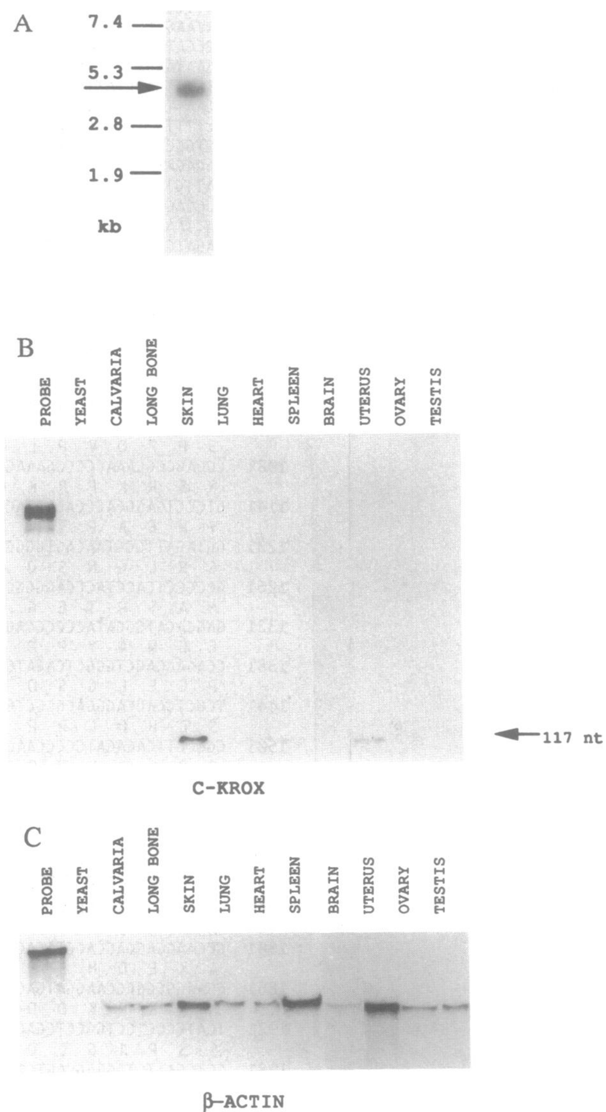

FIG. 3. Analysis of c-Krox gene expression. (*A*) Poly(A)$^+$ RNA (5 μg per lane) from NIH 3T3 fibroblasts analyzed by Northern blot hybridization using as a probe a 410-bp fragment located 5′ of the zinc finger-coding region of the c-Krox cDNA. (*B*) Analysis of c-Krox expression in mouse tissues by RNase protection assay. Samples (10 μg) of total RNA from various mouse tissues were hybridized with a labeled c-Krox cRNA probe. The undigested antisense probe is 160 nt long; the 117-nt protected fragment is denoted by an arrow. Yeast tRNA was used as a negative control. (*C*) Samples (1 μg) of total RNA collected from different mouse tissues were hybridized with a β-actin cRNA as a control. The undigested probe is 300 nt long; the expected protected fragment is 250 nt.

was cloned in both orientations into the *Eco*RI site of pRmHa3 expression vector (13).

RESULTS

Isolation and Sequence Analysis of c-Krox cDNA. An NIH 3T3 fibroblast cDNA expression library in λgt11 was screened with a probe containing four tandem copies of the Aα1(I) binding site. One positive clone, MM13, was isolated and sequenced in its entirety; it contained an open reading frame of 1140 nt. A full-length cDNA clone was later isolated by additional screenings of the NIH 3T3 cDNA library. The DNA sequence analysis of this cDNA revealed an open reading frame with the potential to encode a 427-aa polypeptide of 47,080 Da (Fig. 1*A*). The AUG at the beginning of the open reading frame was in an appropriate context for translation initiation, with upstream termination codons in all three reading frames (14).

The predicted amino acid sequence contained three zinc fingers of the Cys_2His_2 type near the carboxyl terminus (15). Within the zinc finger domain the percentage of similarity between the protein we isolated and Krüppel, a *Drosophila* transcription factor, is 56% (16, 17). In this domain the percentage of similarity between Krox-20, Krox-24, and Krox-2—three mouse homologues of Krüppel (7, 18, 19)—and c-Krox was 55–60% (Fig. 1*B*). We therefore designated

this protein c-Krox, for collagen Krox protein. The amino-terminal region of c-Krox is rich in proline and glutamic acid and resembles the activation domain present in other transcription factors (19, 20). Computer searches of the GenBank/EMBL data bank (166/8386) did not reveal similarities to any other protein sequence outside the zinc finger domain.

c-Krox Protein Binds to the Aα1(I) and Bα1(I) Elements of the Mouse α1(I) Gene Promoter. c-Krox protein was expressed in bacteria as a His_6/c-Krox fusion protein, purified by affinity chromatography (9), and used in gel retardation assays. In direct binding experiments the purified histidine-tagged c-Krox protein bound to either the labeled wild-type Aα1(I) oligonucleotide or the labeled wild-type Bα1(I) oligonucleotide but not to the labeled mutant Aα1(I) or Bα1(I) oligonucleotides (5) (Fig. 2*A*, lanes 1–4). The better affinity of c-Krox for the Aα1(I) oligonucleotide versus the Bα1(I) oligonucleotide is in agreement with what was observed for nuclear extract of NIH 3T3 fibroblasts (5) and may be explained by differences in surrounding sequences. In addition, c-Krox did not bind to a labeled double-stranded oligonucleotide containing the binding site for Sp1, an unrelated zinc finger transcription factor (20) (Fig. 2*A*, lane 5). We do not know the significance of the doublet we observed. In competition experiments, the binding of His_6/c-Krox protein to the labeled wild-type Aα1(I) oligonucleotide was blocked by an excess of unlabeled wild-type Aα1(I) oligonucleotide (Fig. 2*B*, lanes 2–4), but not by the same molar excess of either a mutant Aα1(I) oligonucleotide or an oligonucleotide containing the Sp1 binding site (Fig. 2*B*, lanes 5–7 and 9–11, respectively). These results indicate that c-Krox protein binds specifically to the Aα1(I) and Bα1(I) sites present in the promoter region of the mouse α1(I) collagen gene.

Using the gel retardation assay, we examined the mobility of the DNA–protein complex formed upon incubation of either c-Krox or NIH 3T3 nuclear extract with the labeled Aα1(I) oligonucleotide. The NIH 3T3 nuclear extract protein–DNA complex migrated with a mobility similar to that of the c-Krox–DNA complex (Fig. 2*A*, lane 7 versus lane 6). Further, the binding of the factor present in NIH 3T3 nuclear extract to the labeled Aα1(I) oligonucleotide could be prevented by high concentrations of EDTA (Fig. 2*C*, lanes 1–5) and restored by the addition of zinc ions (Fig. 2*C*, lanes 6–10).

c-Krox mRNA Is Enriched in Skin. Northern blot analysis in which the probe was the 5′ end of c-Krox cDNA, which does not cross-hybridize with other Krox gene products, detected a transcript of ≈3.5 kb in NIH 3T3 cells (Fig. 3*A*). To determine c-Krox mRNA steady-state accumulation in mouse tissues, an RNase protection assay was performed. Interestingly, c-Krox mRNA was detected at the highest level in skin, one of the two major type I collagen-producing tissues (Fig. 3*B*). c-Krox expression could not be detected in bone (the other main type I collagen-producing tissue), brain, lung, or testis (Fig. 3*B*). c-Krox mRNA was also detectable, at a lower level, in heart (an organ containing type I collagen-producing fibroblasts), smooth muscle, liver (data not shown), and spleen. Comparable results were obtained with two different preparations of RNA.

c-Krox Activates Transcription of a Minimal α1(I) Promoter Through the Aα1(I) Binding Site. The transcriptional function of c-Krox was studied by DNA transfection experiments with NIH 3T3 fibroblasts and *Drosophila* S2 cells. In NIH 3T3 cells, cotransfection of an expression vector containing c-Krox cDNA with pK1A4Luc, a reporter vector containing four copies of the Aα1(I) cis-acting element upstream of a minimum α1(I) promoter fused to a luciferase reporter gene, induced a 3-fold increase in luciferase activity, indicating that c-Krox can act as an activator of transcription in this assay. The specificity of this effect was further demonstrated by introducing into the Aα1(I) element mutations that abolish c-Krox binding (pK1A4mLuc). This mutation dramatically reduced the activity of the promoter, suggesting that there is a c-Krox-like activity in NIH 3T3 cells, and also abolished responsiveness to c-Krox (Fig. 4*A*). The same effects were observed whether we used 9 μg or 4.5 μg of expression vector. In *Drosophila* S2 cells, which do not express c-Krox gene, the activity of pK1A4Luc was increased 5-fold upon cotransfection with an expression vector containing c-Krox cDNA in the correct orientation but was unchanged upon cotransfection with an expression vector containing c-Krox cDNA in the opposite orientation (Fig. 4*B*).

DISCUSSION

We have identified a member of the Krox family of transcription factors, termed c-Krox, that regulates type I collagen gene expression. The Krox genes are mouse homologues of the *Drosophila* Krüppel segmentation gene. Krüppel encodes a zinc finger protein that can act as a

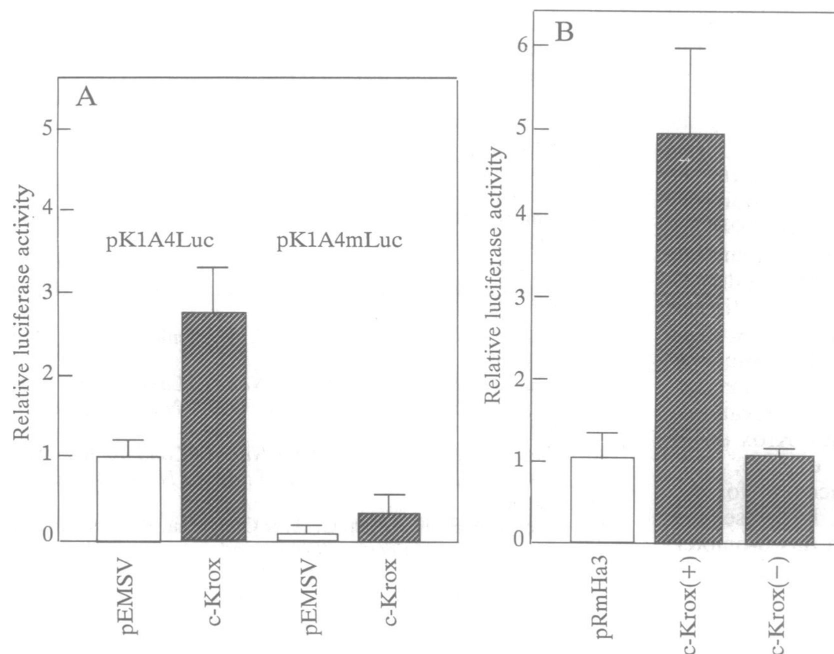

FIG. 4. c-Krox activates transcription through the Aα1(I) binding site. (*A*) NIH 3T3 fibroblasts were transiently transfected with 9 μg of reporter plasmid (pK1Luc, or pK1A4Luc, or pK1A4mLuc), along with 9 μg of pEMSV expression vector that either did (c-Krox) or did not (pEMSV) contain the c-Krox cDNA. A β-galactosidase expression vector, pSVβgal (2 μg) (32), was used as an internal control for transfection. (*B*) *Drosophila* S2 cells were transfected with pK1A4Luc (9 μg) and the pRmHa3 expression vector (9 μg) without cDNA insert (pRmHa3) or containing c-Krox cDNA in the correct orientation [c-Krox(+)] or in the opposite orientation [c-Krox(−)], along with *Drosophila* β-actin–β-galactosidase (32) (5 μg). Bars represent mean and SEM of three independent transfections.

transcription repressor (21). The Krox family includes >20 genes; based on the presence of zinc finger domains it is assumed that they encode transcription factors. To date, however, the existence of a target gene has been documented for only one gene product of this family, Krox-20. Krox-20, whose expression is restricted to and required for the formation of rhombomeres 3 and 5 of the developing hindbrain, regulates HoxB2 expression (22). Thus, c-Krox is a second member of this transcription-factor family for which a target gene has been identified.

Several features distinguish c-Krox from the other Krox gene products, such as Krox-20 and Krox-24, that have been extensively studied. The amino acid sequence of the zinc fingers of c-Krox is different from the sequence of the same domains of Krox-20 or Krox-24 (19). We believe that this difference most likely accounts for the differences in their DNA-binding recognition sequences (23). The spatial pattern of expression of the c-Krox gene is also different from the pattern of expression of Krox-20 and Krox-24. Unlike the latter two genes, which are highly expressed in brain (19, 22), c-Krox transcripts could not be detected in adult brain.

Consonant with its possible role in the regulation of type I collagen gene expression, the c-Krox gene is highly expressed in skin. Preliminary *in situ* hybridization experiments indicate that c-Krox gene expression is restricted to dermis and perichondrium, two tissues highly enriched in fibroblasts (M. Solursh and G.K., unpublished observations). Interestingly, no c-Krox gene expression was detected in bone, the other major type I collagen-producing organ. The skin-specific expression of c-Krox suggests that the mechanisms of cell-specific expression of type I collagen genes are different in osteoblasts and fibroblasts and that c-Krox may play a role in the increase in type I collagen gene transcription observed in fibrotic skin diseases such as scleroderma (24). The expression of c-Krox in muscle tissues suggests that c-Krox may also regulate muscle-specific genes. Consistent with this hypothesis, a c-Krox specific binding site is present in the enhancer of the muscle-specific creatine kinase gene (G.K. and E. Olson, unpublished observation).

To date, two Krox genes have been found to play an important role during early mouse development. Gene targeting experiments have demonstrated that Krox-20 plays an essential role during hindbrain development (25), and Krox-24 is critical for differentiation of myeloblasts along the macrophage lineage (26). In light of these data and of the c-Krox pattern of expression in the adult animal, it will be important to determine precisely the c-Krox pattern of expression during development and in particular to study its function in differentiating fibroblasts.

Both Krüppel and YY1, a human homologue of Krüppel (27), can act as repressors or activators of transcription (21, 22, 27–29). Moreover, Krüppel and YY1 can bind DNA as homodimers or heterodimers (30, 31), thereby expanding their regulatory potential. The ability of YY1 to heterodimerize with Sp1, an activator of transcription, is of particular interest, since an Sp1-like protein binds to the mouse α1(I) collagen promoter close to the c-Krox binding site and participates in the regulation of type I collagen gene expression (5) (C. Desbois and G.K., unpublished observation). Although there is no evidence at present that c-Krox could dimerize, it is a reasonable possibility to explore. This possible dimerization of c-Krox may also account for the doublet we observed in DNA-binding assay. Likewise, the existence of at least one other binding site in this promoter may further broaden the possible regulatory functions of c-Krox. We speculate that the possible interaction of c-Krox with other transcriptional regulators could explain the positive and negative controls of transcription for different genes.

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