Clox, a mammalian homeobox gene related to *Drosophila cut*, encodes DNA-binding regulatory proteins differentially expressed during development

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Summary

We report the isolation of a cDNA encoding a mammalian homeoprotein related to the *Drosophila cut* gene product, called Clox, for Cut like homeobox. In addition to the homeodomain, three 73-amino acid repeats, the so-called *cut* repeats, are also conserved between Cut and the mammalian counterpart described here. This conservation suggests that the *cut* repeat motif may define a new class of homeoproteins. Both cloned and endogenous Clox proteins are nuclear DNA-binding proteins with very similar sequence specificity. Western blot analysis revealed several distinct Clox protein species in a variety of tissues and cell types. The relative abundance of these proteins is regulated during mouse development and cell differentiation in culture. Interestingly, ~180-190×10^3 M_r Clox proteins predominate in early embryos and are upregulated in committed myoblasts and chondrocytes, but downregulated upon terminal differentiation. Clox DNA-binding activity is correlated with the abundance of these proteins. In contrast, larger Clox protein species (~230-250×10^3 M_r) are detected mainly in adult tissues and in terminally differentiated cells. Cotransfection experiments show that Clox proteins can function as repressors of tissue-specific gene transcription. Thus, Clox, like their *Drosophila* counterparts, are candidate regulators of cell-fate specification in diverse differentiation programs.

Key words: *Clox*, homeobox genes, DNA binding-proteins, transcription factors, mouse development.

Introduction

Molecular and genetic analyses have demonstrated that homeobox-containing genes play a key role in the developmental process of segmentation and specification of individual segments during *Drosophila* embryogenesis (for reviews, see Scott et al., 1989; Hayashi and Scott, 1990). In higher animals, the temporal and spatial patterns of expression of homeobox genes suggest that, as in *Drosophila*, they play an important role in the establishment of the general body plan and cell-fate determination during embryonic development (reviewed by Holland and Hogan, 1988; Shashikant et al., 1991; McGinnis and Krumlauf, 1992). The phenotypes resulting from alterations of homeobox gene expression in *Xenopus* embryos and transgenic mice are consistent with such a role (McGinnis and Krumlauf, 1992). However, with few exceptions, such as the POU-domain (for reviews, see Hayashi and Scott, 1990; Rosenfeld, 1991; Ruvkun and Finney, 1991; Schöler, 1991; Shashikant et al., 1991) and the HOX3 and HOX4 homeoproteins (Zappavigna et al., 1991; Arcioni et al., 1992), the DNA-binding activities of mammalian homeobox gene products and their transcriptional regulatory functions remains to be established.

The *Drosophila cut* locus provides an intriguing model to study tissue-specific gene regulation, because different mutations in *cut* affect diverse tissues (Johnson and Judd, 1979; Jack, 1985; Bodmer et al., 1987; Liu et al., 1991). The product from the *cut* locus has been cloned and shown to encode a large homeodomain nuclear protein (Cut) (Blochlinger et al., 1988). Cut has been detected in external sensory (es) organs but not in internal chordotonal (ch) organs (Blochlinger et al., 1988). Embryonic lethal *cut* mutations cause the transformation of es into ch organs (Bodmer et al., 1987). Conversely, ectopic expression of Cut in *Drosophila* embryos specifically transforms ch organs into es organs (Blochlinger et al., 1991). On the basis of these results, it has been suggested that Cut functions as a determination factor that specifies sensory organ identity in precursor cells. Evidence suggests that *cut* is also involved in cell type specification of Malpighian tubules (Liu et al., 1991). The Cut protein has several characteristic features, including the presence of three novel internal repeats (*cut* repeats) and a highly divergent homeodomain. Additionally, *cut* shows a cell-specific pattern of expression rather than the spatial-specific pattern of expression characteristic of many homeotic genes (Shashikant et al., 1991; McGinnis and Krumlauf, 1992). Together, these features suggest that Cut may define a new family of homeoproteins. While this manuscript was in preparation, a human

counterpart of Cut, called CDP, has been isolated (Neufeld et al., 1992). However, no direct evidence for a role of Cut or CDP as transcriptional factors has been reported.

We describe the isolation of cDNA clones encoding a mammalian homeoprotein that is closely related to Cut and CDP, named Clox (for Cut-like homeobox). Clox contains the three diagnostic internal *cut* repeats and a homeodomain that is more closely related to that of Cut and CDP than to other homeoproteins. Clox comprises a family of nuclear proteins that display developmentally regulated patterns of expression and DNA-binding activities specific to the differentiation stage. Like their *Drosophila* counterparts, Clox proteins are expressed in several neural and nonneural tissues. Transient expression assays show that Clox may function as repressors of gene transcription. These observations suggest that *Clox* gene products are transcriptional factors important for cell-fate determination and differentiation during mammalian embryonic development.

Materials and methods

Cell culture

HeLa, Cos, CV1 and C3H10T1/2 clone 8 (Reznikoff et al., 1973) cells were passed in Dulbecco's modified Eagle's medium + 10% fetal bovine serum at subconfluent densities. Sol8 myogenic cells (Mulle et al., 1988) were cultured as described (Thompson et al., 1991). RCJ 3.1C5 (RCJ) chondrogenic cells were maintained either in standard medium (-Minimum Essential Medium + 15% fetal bovine serum) or differentiation medium (standard medium supplemented with 10 mM sodium -glycerolphosphate, 50 μg/ml ascorbic acid, and 100 nM dexamethasone; Grigoriadis et al., 1989, 1990). RCJ cells maintained in standard medium could be referred to as undifferentiated chondroblast progenitors, since they were pleomorphic and did not develop significant cartilage nodules (Grigoriadis et al., 1989). However, when switched to differentiated medium, they became cuboidal and developed discrete cartilage nodules. Under these conditions, RCJ cells have been shown to stain with antibodies against collagen II (Grigoriadis et al., 1989).

cDNA cloning and sequencing

A gt11 cDNA library from adult dog cardiac ventricle (Scott et al., 1988) was screened using a randomly ligated DNA probe consisting of the e2 subelement from the rat -MHC gene 5 -upstream sequences, position −288 to −267 (Thompson et al., 1991), along with cohesive *Bgl*II sites. To prepare the catenated probe, the ends of the monomer fragment were filled in with [-^{32}P]dATP, dCTP, dGTP and dTTP using Klenow enzyme, and the resulting 31 base pair blunt-ended DNA fragment, 5 -GATCT-GTGAGCTGTGGAATGTAAGGGAGATC-3 (denoted e2 or 1 × probe), was ligated with T4 DNA ligase. Lytic growth of bacteriophage gt11 in *E.coli* Y1090 and transfer of -galactosidase fusion proteins to duplicate nitrocellulose filters impregnated with IPTG was carried out according to Young and Davies (1983). Subsequent treatment of the filters and incubation with the catenated probe were carried out essentially as described by Singh et al. (1988). In a screen of 5 × 10^5 recombinants, one positive phage, designated Clox1, was isolated. Screening of the corresponding gt10 library with   Clox1-cDNA as probe using standard procedures (Sambrook et al., 1989) allowed the isolation of four additional positive phages (see Fig. 2A).

Clox cDNAs were subcloned into pGEM3 (Promega) and sequenced with Sequenase (US Biochemicals), using universal primers. Some DNAs were cloned into pGEM7zf(+) to generate deletions with Exonuclease III and rescue single-stranded DNA as recommended by the manufacturer (Promega). Taq polymerase (US Biochemicals) and deoxyinosine nucleotide analogs were used to reveal and resolve gel compressions in the G-C rich regions.

Eukaryotic Clox expression systems

A composite Clox cDNA was made by ligating an *Eco*RI/*Bgl*II fragment including nucleotides 1-1130 to a *Bgl*II/*Kpn*I fragment containing nucleotides 1131-3379 (excised from pGEM3-Clox2 and pGEM3-Clox3, respectively; see Fig. 2A). The resulting *Eco*RI/*Kpn*I fragment was subcloned into pGEM7zf(+). pGEM3-Clox1 (amino acids 419-975, see Fig. 2A) was also used as template for in vitro transcription. RNA synthesis and in vitro translation with rabbit reticulocyte lysate was performed using 1 μg of linearized plasmid and 10 units of T7 RNA polymerase as suggested by the manufacturers (Stratagene; Promega).

Sense and antisense eukaryotic Clox expression vectors were constructed by subcloning into the unique *Eco*RI site of pMT2 (Kaufman et al., 1989) the composite Clox cDNA or Clox1-cDNA (see Fig. 2A). The pMT2-MEF2 vector is described in Yu et al. (1992). The chloramphenicol acetyl-transferase (CAT) reporter constructs, [e2]x3(rev) TK and [−285/−180] A10, and the procedure for transient transfections, have been previously described (Thompson et al., 1991). For immunocytochemistry, Cos cells growing on glass coverslips were transfected with pMT2-Clox-sense vectors. For the Sol8 cotransfections, 5 μg of CAT reporter construct and the indicated amount of pMT2-Clox1 (sense or antisense) were transfected. For the CV1 cotransfections, 10 μg of CAT reporter construct and 5 μg of the indicated pMT2 constructs were transfected. In all cases, pMT2 control vector was added to have constant 20 μg of DNA per assay. Cells were cotransfected with 3 μg of murine leukemia virus (MLV) -galactosidase plasmid to control for transfection efficiency.

Antisera production

Rabbit immunization and purification of immunoglobulins by binding to protein A-Sepharose beads (Repligen) were carried out using standard procedures (Harlow and Lane, 1988). A peptide corresponding to amino acids 636-651 of the predicted Clox protein was selected for antibody production based on its hydrophilicity characteristics (Hopp and Woods, 1981) and a low level of similarity with known peptide sequences (IG-Suite, Intelligenetics). An amino terminal cysteinyl residue was added for consequent conjugation to BSA and keyhole limpet hemocyanin via maleimidobenzoyl sulfosuccinimide ester. Antibodies were generated using 2.5 mg of conjugated peptide/injection. Some anti-peptide (-P) serum was further affinity purified by passaging over a 2 ml column of Affi-Gel 10 (Bio-Rad) that had been crosslinked to immunogenic peptide.

A GST-Clox fusion protein was made as follows (see Fig. 2A). A *Bam*HI-cDNA restriction fragment encoding amino acids 419-740 of the predicted Clox protein was excised from pGEM3-Clox1 and subcloned in the bacterial expression vector pGEX-2T (Glutagene^{TM}). The fusion protein was overexpressed in *E. coli* (DH5) and purified essentially as described by Smith and Johnson (1988). To raise the -FP antiserum, a gel slice containing the fusion protein (~73×10^3 M_r) was excised, cut in small fragments and injected to rabbits (0.1-0.2 mg protein/injection).

Immunocytochemistry

CD1 albino mice were obtained from Charles River. Staged mouse embryos (the middle of the night of mating was designated as day 0 of pregnancy) were dissected in ice-cold phosphate-buffered saline (PBS) and subsequently embedded in OCT compound

(Miles Laboratories) and frozen on dry ice. Frozen sections (15 µm) were mounted on glass slides previously coated with 1% gelatin. All incubations described below were done at room temperature in a humid chamber and were followed by washes with PBS. The sections were fixed for 3 minutes with methanol (−20ºC), permeabilized for 5 minutes with 0.25% NP-40 in PBS and blocked for 30 minutes in PBS containing 2% normal goat serum, 0.1% NP-40. Incubations with affinity-purified -P antibodies diluted to 10 µg/ml with blocking buffer were carried out for 1.5 hours. For peptide competition, 14 µM of immunogenic peptide was added to the diluted antibodies and rocked for 45-60 minutes prior to incubation. Sections were incubated in the dark for 45-60 minutes with fluorescein-conjugated goat anti-rabbit IgG (1:600, Cappel), and nuclei were counterstained with Hoechst 33258 (1 µg/ml in PBS) for 1-2 minutes before mounting the sections in 1 mg/ml p-phenylenediamine dihydrochloride, 90% glycerol/PBS. Sections adjacent to those used for immunocytochemistry were stained with cresyl violet for routine histology.

For immunofluorescence, cells growing on glass coverslips were washed three times in PBS, fixed for 10 minutes in PBS containing 2% formaldehyde (freshly prepared from paraformaldehyde), and treated as indicated above. Cells and tissues were visualized with incident light fluorescence (495 nm) on a Zeiss Axiophot photomicroscope (Thornwood, NY) equipped with Plan-Neofluar objectives. Fluorescent images were recorded on Kodak (Rochester, NY) T-Max film (400 ASA). -FP antibodies did not react in immunocytochemistry.

Protein extracts, western and dot blot analysis and immunoprecipitation

Mouse embryos and organs were removed and quickly frozen into liquid nitrogen. The developing vertebral column and ribs were the source of cartilage in 15- and 17-day-old embryos, whereas the xiphoid process was dissected in newborn and adult mice. Mouse tissues pooled from 2-8 different animals were lysed by sonication at 4ºC in 4 volumes of PBS containing 15% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 10 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptine, and 5 µg/ml aprotinin. The sonicated samples were allowed to incubate on ice for 15-30 minutes and then spun at 16,000 g for 10 minutes at 4ºC to remove insoluble material. For whole cell extract preparation, cells were washed three times in PBS, collected by centrifugation and resuspended in 1 volume of ice-cold 2 × lysis buffer [20 mM Hepes-KOH, (pH 7.8), 0.6 M KCl, 1 mM DTT, 20% glycerol, 2 mM EDTA, 2 µg/ml leupeptine]. Cells were lysed with three cycles of freezing and thawing. To remove insoluble material, extracts were spun at 16,000 g for 20 minutes at 4ºC. Protein concentration was determined using the modified Bradford assay (Bio-Rad).

For Western blot, 50 µg of protein was separated by electrophoresis through 6.5% SDS-polyacrylamide gels (Laemmli, 1970) and transferred by semidry blotting to Immobilon-P (0.45 µm, Millipore) using 10 mM CAPS (pH 11), 10% methanol. For dot blot, samples were applied to nitrocellulose (0.45 µm, MSI). Filters were blocked for 1 hour at 37ºC with 25 mM Tris-Cl (pH 8.0), 125 mM NaCl, 0.05% Tween-20 (TBST), containing 4% nonfat dry milk. Incubations with -P and -FP antibodies diluted to 4-7 µg/ml with TBST, 2% nonfat milk were performed for 8-12 hours at 4ºC. Washes, incubations with secondary antibody and visualization of the immune complexes were carried out as recommended by the manufacturer (Enhanced Chemiluminiscence Kit, Amersham).

For immunoprecipitation, 0.5 ml RIPA buffer [10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS], 40 µl of a slurry of 50% protein A-Sepharose beads (in RIPA buffer) and 5 µl of -P or preimmune serum were rocked

at room temperature for 30 minutes. For peptide competition, 46 µM of immunogenic peptide was added to the incubation mixture. ³⁵S-labeled in vitro translation products (7 µl, see above) were then added, and the reaction mixtures were rocked at 4ºC for 2.5 hours. Immunoprecipitates were washed four times with RIPA buffer before being loaded onto 6.5% SDS-polyacrylamide gels (Laemmli, 1970). Gels were fixed in 10% (vol./vol.) methanol, 10% (vol./vol.) acetic acid and dried before autoradiography.

Gel mobility shift assays (GMSA)

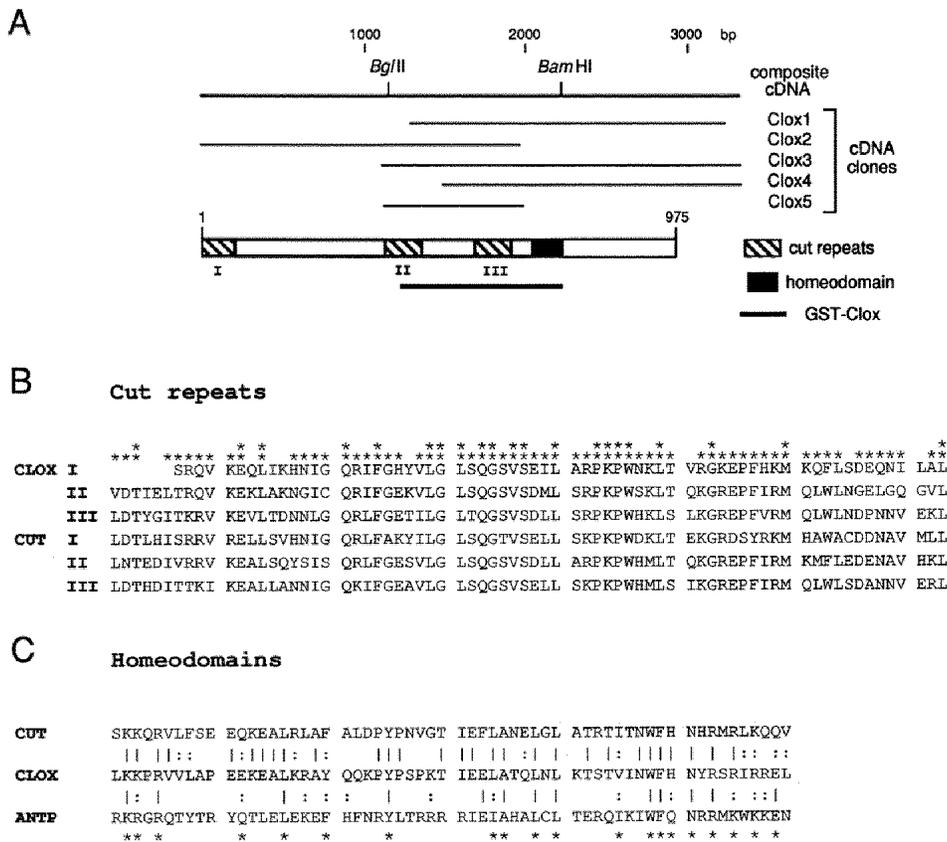Oligonucleotides consisting of two copies of the e2 probe (see above) were synthesized and named Cbs-1 (5 -3/5 -3) and Cbs-2 (3 -5 /5 -3). Cbs-3 consisted of the sequence from −354 to −269 of the rat -MHC enhancer, which contains a single copy of the e2 element (Thompson et al., 1991). Oligonucleotides were end labeled using T4 kinase and [-³²P]ATP (3000 Ci/mM) and gel purified. For GMSA, whole cell extracts (15-20 µg), in vitro translation products (5 µl), or GST-Clox fusion protein (4 µg), were preincubated on ice for 10 minutes with 50 µg/ml poly(dI-dC) in binding buffer [20 mM Hepes-KOH (pH 7.8), 50 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.5 mM MgCl₂]. 20 fmol of probe, simultaneously with unlabeled competitor where indicated, was then added for a final reaction volume of 20 µl. Incubation on ice was continued for 30 minutes and the samples were electrophoresed at 4ºC in 0.5 × TBE, 3.5% native polyacrilamide gels (80:1 acryl/bis). For GMSA involving the addition of antibody, extracts were incubated at 37ºC for 15 minutes simultaneously with -P antiserum or preimmune serum where indicated (3.5 µg immunoglobulins). Then poly(dI-dC) and binding buffer were added, and the samples allowed to incubate on ice for an additional 10 minutes before the addition of probe.

Results

Isolation of Clox cDNAs and predicted protein sequence

The Clox1 cDNA was initially isolated from a dog heart gt11 expression library, in an attempt to clone a cardiac transcription factor that interacts with the e2 subelement of the sarcomeric -myosin heavy chain (-MHC) enhancer. This subelement confers muscle-specific and developmentally regulated expression to a heterologous promoter (Thompson et al., 1991 and see Material and methods). Subsequently, the Clox1 cDNA was used as probe to isolate four additional partially overlapping clones. The ~3.4 kb Clox cDNA sequence predicted an open reading frame which encodes a polypeptide of 975 amino acids (Figs 1, 2A). Three independent cDNA clones confirmed an in-frame TGA stop codon (nucleotides 2926-2928), and two clones containing the 3 -most region of the composite cDNA showed a putative AATAA polyadenylation signal and the poly(A) tract. Attempts to 5 -extend the Clox sequence further were unsuccessful. The predicted Clox polypeptide bears a homeodomain with 48% amino acid identity to that of the *Drosophila cut* gene product (Blochlinger et al., 1988) (Fig. 2C). In contrast, there is only 22% amino acid identity between the homeodomain of Clox and Antennapedia (Antp), the archetype homeoprotein (Schneuwly et al., 1986). The nine amino acids conserved in most of the homeodomains so far characterized are also maintained in Clox, except for a Gln → Glu substitution at position 12, which has been also changed to Ala

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tcccggcaggtgaaggaacagttgatcaagcacaatattggacagcgcattttcggacattatgtcttgggactgtctcaagggtctgtgagcgagattctggcccggcccaagccatgg   120
 S  R  Q  V  K  E  Q  L  I  K  H  N  I  G  Q  R  I  F  G  H  Y  V  L  G  L  S  Q  G  S  V  S  E  I  L  A  R  P  K  P  W    40

aataaactgaccgttcgcggcaaggagccctttcacaagatgaagcagttcctctcggatgagcaaaacatcttggctcttcgcagcatccaaggcagacaaagagagaatccaggccag   240
 N  K  L  T  V  R  G  K  E  P  F  H  K  M  K  Q  F  L  S  D  E  Q  N  I  L  A  L  R  S  I  Q  G  R  Q  R  E  N  P  G  Q    80

agcctgaacgactatttcaggaagtaccgaaacgaagaaatgggtccgaaggtaacatcacaacccggatccgagcctccgagaccggatccgacgaagcaatcaagtccatcctggaa   360
 S  L  N  R  L  F  Q  E  V  P  K  R  R  N  G  S  E  G  N  I  T  T  R  I  R  A  S  E  T  G  S  D  E  A  I  K  S  I  L  E   120

caagccaaaagggagctgcaggtccagaaaactgcagagccggcccagccttcttccacgtccagcagcgggacgtcagacgacgccatccgctccatcctgcagcaggccgccgcgag   480
 Q  A  K  R  E  L  Q  V  Q  K  T  A  E  P  A  Q  P  S  S  T  S  S  S  G  T  S  D  D  A  I  R  S  I  L  Q  Q  A  R  R  E   160

atggaggccagcaggccgccctcgaccctgccttaaagcccgcaccgctgtcccaggctgacctcgccatcctgagccccaagttgataccttcctcacccatgtcgtctgtgtccagc   600
 M  E  A  Q  Q  A  A  L  D  P  A  L  K  P  A  P  L  S  Q  A  D  L  A  I  L  S  P  K  L  I  P  S  S  P  M  S  S  V  S  S   200

taccctccactcgccctgtccctgaagaagcccccacgcccctgacaccagcgcctccaccctgcccaaccccccagccctcaaaaaggagtcccaggatgcgcccgggctggacctc   720
 Y  P  P  L  A  L  S  L  K  K  P  P  T  A  P  D  T  S  A  S  T  L  P  N  P  P  A  L  K  K  E  S  Q  D  A  P  G  L  D  L   240

ccgggagcggctgagtctgcacagggggtcctgcggcacgtgaagagcgagctgggccgcagcggtgtgtggaaggaccactggtggagcacgtccagccagagaggaagagcgccgca   840
 P  G  A  A  E  S  A  Q  G  V  L  R  H  V  K  S  E  L  G  R  S  G  V  W  K  D  H  W  W  S  T  V  Q  P  E  R  K  S  A  A   280

cccccgaggacgccaagtcagaagaagccggtggcaccaaggagaaaggggggcggccaaggccacgggccgatcgcagccagctccagggaccctcatcatcgtcggagtactggaagg   960
 P  P  E  D  A  K  S  E  E  A  G  G  T  K  E  K  G  G  G  Q  G  H  G  P  I  A  A  S  S  R  D  P  H  H  R  R  S  T  G  R   320

aatggcccagcgctgagtccgcgtactccccagagctctgagctgagcctgactggagccagccgcagcgagacaccccagaacagcccctgccctcctccccgattgtgcccatgtcc  1080
 N  G  P  A  L  S  P  R  T  P  Q  S  S  E  L  S  L  T  G  A  S  R  S  E  T  P  Q  N  S  P  L  P  S  S  P  I  V  P  M  S   360

aagcccgccaagccctcggtccctccactgaccccggagcagtatgagatctacatgtaccaggaggtggacaccattgagctcactcggcaggtgaaggagaagctggccaagaacggc  1200
 K  P  A  K  P  S  V  P  P  L  T  P  E  Q  Y  E  I  Y  M  Y  Q  E  V  D  T  I  E  L  T  R  Q  V  K  E  K  L  A  K  N  G   400

atctgtcaaagaatcttcgggggagaaggtactgggccctgtcccagggcagcgtcagcgacatgctgtcccggccaaaagccttggagcaagctgacccagaaaggccgtgaacccttcatc  1320
 I  C  Q  R  I  F  G  E  K  V  L  G  L  S  Q  G  S  V  S  D  M  L  S  R  P  K  P  W  S  K  L  T  Q  K  G  R  E  P  F  I   440

cggatgcagctctctggctcaacggcgagctgggccagggtgtcctgcctgtccagggtcagcagcaagggccagttctccattctgtgacgtcactacaggaccccctgcagcaggctgt  1440
 R  M  Q  L  W  L  N  G  E  L  G  Q  G  V  L  P  V  Q  G  Q  Q  Q  G  P  V  L  H  S  V  T  S  L  Q  D  P  L  Q  Q  G  C   480

gtgagctcagaaagcactccaaagacctctgctagctgcagccctgcacctgaatcgcgatgagctctagtgaatccgtgaagagtttgactgaacttgtccagcagccctgtcccccc  1560
 V  S  S  E  S  T  P  K  T  S  A  S  C  S  P  A  P  E  S  P  M  S  S  S  E  S  V  K  S  L  T  E  L  V  Q  Q  P  C  P  P   520

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gccatgtctccagagctggacacctacggtataaccaagagagtcaaggaggtgctgacggacaacaatctcggtcagcgcttattcggggagaccatttttaggcctcacccaaggctcc  1800
 A  M  S  P  E  L  D  T  Y  G  I  T  K  R  V  K  E  V  L  T  D  N  N  L  G  Q  R  L  F  G  E  T  I  L  G  L  T  Q  G  S   600

gtctccgacctcctctcccgtccaaagccctggcacaagctcagtctgaagggacgggagcctttgtccggatgcagctatggctgaatgacccaacaacgtggagaagctgatggac  1920
 V  S  D  L  L  S  R  P  K  P  W  H  K  L  S  L  K  G  R  E  P  F  V  R  M  Q  L  W  L  N  D  P  N  N  V  E  K  L  M  D   640

atgaaacgcatggagaagaaagcatacatgaagcggcggcacagctcggtcagtgacagccagccctgcgagcccccctccgtgggcatcgactacagccaggcgccagcccccagccc  2040
 M  K  R  M  E  K  K  A  Y  M  K  R  R  H  S  S  V  S  D  S  Q  P  C  E  P  P  S  V  G  I  D  Y  S  Q  G  A  S  P  Q  P   680

cagcaccagctgaagaaacccgagtggtgctggctcctgaagagaaagaagctctgaaacgagcttatcagcaaaagccataccgtcaccaaaaaccatcgaagaactcgccacccag  2160
 Q  H  Q  L  K  K  P  R  V  V  L  A  P  E  E  K  E  A  L  K  R  A  Y  Q  Q  K  P  Y  P  S  P  K  T  I  E  E  L  A  T  Q   720

ctcaacttgaaaaccagcaccgtcatcaactggttccacaattacaggtctcggatccgcagagaactgttcattgaggagattcaggccgggagccagggccagggccggggccaggcac  2280
 L  N  L  K  T  S  T  V  I  N  W  F  H  N  Y  R  S  R  I  R  R  E  L  F  I  E  E  I  Q  A  G  S  Q  G  Q  A  G  A  R  H   760

tccccgtcgccgaagcagcggcgcggcccccagctcggagggcgacagctgcgacggcgtggaggccgccgagggcccgggcgccgcggacgccgaggagtcggcgccgcggccgcc  2400
 S  P  S  A  R  S  S  G  A  A  P  S  S  E  G  D  S  C  D  G  V  E  A  A  E  G  P  G  A  A  D  A  E  E  S  A  P  A  A  A   800

gccaagtctcaggagggccagccgaggcggccgtggccccggaggagagcgggaggaggccgcgaggccggcgggagaagcgcagccgcgccctcggggcccggccccggccccggaaga  2520
 A  K  S  Q  G  G  P  A  E  A  A  V  A  P  E  E  R  E  E  A  P  R  P  A  E  K  R  S  R  R  P  R  G  P  G  P  G  P  G  R   840

cgcggaggaggaggcccggccgcccggcgcccccgccgcgccgccgccgcgagggcccagccgacggccgggggccgtgccaaaccccgccgccgccgccgccgcgccgccgccgc  2640
 R  G  G  G  G  P  A  P  G  A  P  A  A  P  A  A  A  A  R  G  P  S  R  R  P  G  A  R  A  K  P  R  R  R  R  R  R  R  R  R   880

cacgccgcggggggaggacgccgctacctcagccgccccgcccgcgggaggcccctgccgggcccgggacggcgccacaggagttccgctttgccaagcaccagcgcgcgcggcggcggc  2760
 H  A  R  G  G  G  R  R  Y  L  S  R  P  A  R  G  G  P  C  R  A  R  D  G  A  H  R  S  S  A  L  P  S  T  S  A  P  A  A  A   920

cgcaggccagctcgctgcagagacctcttcggcctcccggaggcggcggggccgccagggactctcgagacaaccccctgaggaagaagaaggccgcgaacttgaacagcataatccaccgc  2880
 R  R  P  S  S  L  Q  S  L  F  G  L  P  E  A  A  G  A  R  D  S  R  D  N  P  L  R  K  K  A  A  N  L  N  S  I  I  H  R   960

ctggagaaggcgccagccgggaggagcccatcgaatgggagttctgagagggcgcccgccgcgccgggccgggccgggccgagccgcgccgccgccgcgccgggcgggccgagcgggcta  3000
 L  E  K  A  A  S  R  E  E  P  I  E  W  E  F                                                                              975

agcgggccgagccgggccgagtctgccgagccctgccgagtctgaccgagccgagccgggccgaacctggccgagcgggccgggccgggcagaggggcgggcgcggactccgcggcctgg  3120

accgtgttgcgcacttaccgccctgcgggccacagggcaaaatcgcgataggccaaggtgcatatagaaaacaaaggagcattaagcccaatctatgtcgtgttttcaaggaagaaacg  3240

gaaatgtgtagtcgagcctttttgtaccctgaagtgtttttttttattgccctaagtgatttccacaggttctggaataactcttacagctttgccttgcgccctcctctttcgtgtgggc  3360

tttaaaaaaaaaaaaaaaa                                                                                                      3379
```

Fig. 1. Partial Clox nucleotide coding sequence and predicted protein sequence. The homeodomain is underlined; the three *cut* repeats are boxed. Residues in these domains are typed in boldface. The sequence of the peptide used to raise -P antibodies is marked by asterisks (*). An in-frame stop codon and the putative polyadenylation signal at the 3′ end of the transcript are typed in bold. The predominant divergences between Clox and CDP coding regions are found between amino acids 277 to 332 in Clox and 833 to 892 in CDP, and between amino acids 795 to 920 in Clox and 1354 to 1450 in CDP (Neufeld et al., 1992).

A

```
                    1000          2000          3000  bp
                     |             |             |
                    BglII         BamHI
                                                   composite
                                                   cDNA
  ───────────────────────────────────────────────
            ──────────────────────────────────    Clox1
      ────────────────────────────────────        Clox2  ]
         ──────────────────────────────           Clox3  ]  cDNA
              ──────────────────────────────      Clox4  ]  clones
                    ──────────────────            Clox5  ]
   1                                          975
  ┌──────────────────────────────────────────┐
  │▨▨      ▨▨   ▨▨ ■              │
  └──────────────────────────────────────────┘        ▨▨  cut repeats
   I              II   III
                                                       ■   homeodomain
        ────────────────                               ──  GST-Clox
```

B   **Cut repeats**

```
              ***  *****  ** *  ****  * *****  ***  **********  *********  ********  *  **** *****  **
CLOX  I            SRQV  KEQLIKHNIG  QRIFGHYVLG  LSQGSVSEIL  ARPKPWNKLT  VRGKEPFHKM  KQFLSDEQNI  LAL
      II     VDTIELTRQV  KEKLAKNGIC  QRIFGEKVLG  LSQGSVSDML  SRPKPWSKLT  QKGREPFIRM  QLWLNGELGQ  GVL
      III    LDTYGITKRV  KEVLTDNNLG  QRLFGETILG  LTQGSVSDLL  SRPKPWHKLS  LKGREPFVRM  QLWLNDPNNV  EKL
CUT   I      LDTLHISRRV  RELLSVHNIG  QRLFAKYILG  LSQGTVSELL  SKPKPWDKLT  EKGRDSYRKM  HAWACDDNAV  MLL
      II     LNTEDIVRRV  KEALSQYSIS  QRLFGESVLG  LSQGSVSDLL  ARPKPWHMLT  QKGREPFIRM  KMFLEDENAV  HKL
      III    LDTHDITTKI  KEALLANNIG  QKIFGEAVLG  LSQGSVSELL  SKPKPWHMLS  IKGREPFIRM  QLWLSDANNV  ERL
```

C   **Homeodomains**

```
CUT    SKKQRVLFSE  EQKEALRLAF  ALDPYPNVGT  IEFLANELGL  ATRTITNWFH  NHRMRLKQQV
       ||  ||::     |:||||: |:     |||     | || || :| |   | |: |||| | |  |:: ::
CLOX   LKKPRVVLAP  EEKEALKRAY  QQKPYPSPKT  IEELATQLNL  KTSTVINWFH  NYRSRIRREL
       |: |          :   | : :   |   :      |:| | |      :   ||: | | : ::|
ANTP   RKRGRQTYTR  YQTLELEKEF  HFNRYLTRRR  RIEIAHALCL  TERQIKIWFQ  NRRMKWKKEN
       ** *          *   *   *       *       ** *  *      *  *** * * * * *
```

Fig. 2. (A) Schematic representation of the composite Clox cDNA, cDNA Clox clones, and predicted Clox protein. Restriction sites referred to in the text are indicated. The protein sequence is aligned with the cDNA clones. The numbers indicate amino acid residues. The three *cut* repeats are numbered in roman characters. Underlined is the region of Clox contained in the GST-Clox fusion protein. (B) Sequence comparison of Cut and Clox repeats. Single asterisks (*) indicate identical or conservative amino acids in three or more repeats; two asterisks indicate amino acid identity in all *cut* repeats. (C) Sequence comparison of Clox, Cut and Antp homeodomains. The symbols | and : denote, respectively, identical or conservative amino acids. The asterisks indicate amino acid identity or similarity between the three homeodomains. The nine amino acids conserved in most of the homeodomains so far characterized are at positions 5, 12, 16, 25, 40, 48, 49, 51 and 53 (see text).

or Ser in the POU homeodomain (Shashikant et al., 1991). The similarities among three internal repeats in Clox and Cut proteins, the so-called *cut* repeats (Blochlinger et al., 1988), are conspicuous (Fig. 2B). This motif, which has been originally described as a 60-amino acid polypeptide, can be elongated up to 73 amino acids by virtue of the similarities between Clox and Cut. Amino acid identity between these repeats is 54-64% in Clox, 52-63% in Cut, and 50-70% between Clox and Cut. Interestingly, Clox and Cut third repeats are more similar to one another (70%) than to any other repeat. Twenty-one residues (28%) are identical in all repeats. No other significant sequence similarities between Clox and Cut outside their homeodomains and *cut* repeats were found. It is noteworthy to point out, however, the structural similarity between Clox and Cut in the polar polypeptide between the third *cut* repeat and the homeodomain, as revealed by sequence analysis using the PC/Gene software (Intelligenetics). Both peptides have basic theoretical pI (Clox = 9.4, Cut = 10.26), similar hydrophilicity and charged residues pattern, and similar charge profile as a function of pH. This conservation suggests that this region of the protein may play an important structural role.

Recently, CDP, a human counterpart of *cut* has been cloned independently by others (Neufeld et al., 1992). Although Clox and CDP sequences are conserved almost in their entirety, a complete divergence noted in their respective carboxyl terminal coding portions indicates that they correspond to alternatively spliced transcripts (see legend to Fig. 1).

Characterization of anti-Clox antibodies

Two anti-Clox antibodies were generated to investigate the pattern of expression of *Clox* during mouse development (see below). Rabbits were immunized with a Clox-specific peptide and with a GST-Clox fusion protein (see Materials and methods and Figs 1, 2A). GST-Clox was recognized by the F2 anti-Cut antiserum (data not shown; Blochlinger et al., 1990). The specificity of the anti-Clox antibodies was initially tested by dot blot assay (Fig. 3A). Anti-peptide antibodies (-P) specifically recognized both the immunogenic peptide and GST-Clox fusion protein, but not GST. Anti-GST-Clox antibodies (-FP) only recognized GST-Clox fusion protein and not GST or the immunogenic peptide used to raise -P antibodies, although this peptide sequence is contained in GST-Clox. These results were confirmed by ELISA (data not shown). -P antibodies immunoprecipitated in vitro translated Clox products (Fig. 3B). Immunoprecipitated products were not detected when the affinity-purified -P antibody was preincubated with immunogenic peptide, or when preimmune serum was used. Of note, in vitro translated Clox products showed a slower electrophoretic mobility than that expected from the predicted protein sequence, possibly due to the clusters of basic residues observed in the carboxyl terminal portion (see Fig. 1).

Three criteria were used to judge the specificity of the bands detected by western blot (see below, Figs 4 and 9B). First, the absence of signal when using the proper preimmune serum; second, the competition of the signal by the appropriate immunogen (synthetic peptide or fusion pro-
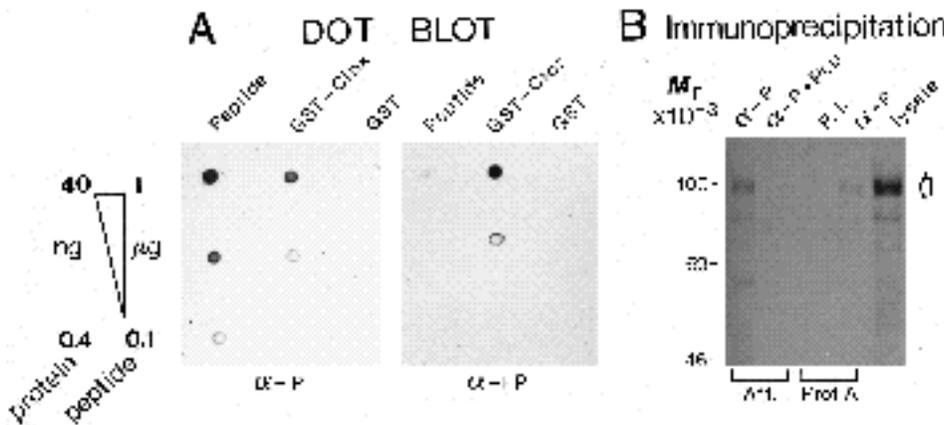
Fig. 3. Specificity of anti-Clox antibodies. (A) Dot blot assay. Increasing amounts of immunogenic peptide and protein (GST or GST-Clox) were blotted as schematized in the left. Filters were incubated with the anti-peptide (α-P) or anti-GST-Clox (α-FP) antibodies (see Materials and methods). No signal was detected in filters incubated with equivalent amounts of the corresponding preimmune serum (not shown). (B) Immunoprecipitation of in vitro translated Clox products with α-P antibodies. pGEM3-Clox1 (see Fig. 2A) was used as template for in vitro transcription/translation. The ~100×10³ in vitro translated protein (arrow) was immunoprecipitated with either affinity-purified antibodies (Aff., α-P lane; 9 μg/ml) or protein A-purified antibodies (Prot A, α-P lane; 17 μg/ml). Controls were: affinity-purified antibodies preincubated with immunogenic peptide (α-P + Pept), and preimmune serum (P.I.; 17 μg/ml).

tein) and third, the ability of both α-P and α-FP to recognize the same proteins (as judged by their relative molecular mass). It should nevertheless be pointed out that the higher relative molecular mass Clox-specific bands (above $200×10^3$; see Figs 4 and 9B) were better recognized by α-P antibodies.

Several Clox proteins are regulated during mouse development

To investigate whether *Clox*, like its *Drosophila* counterpart (Blochlinger et al., 1990), is regulated during embryonic development, we examined its pattern of expression in staged mouse embryos by western blot analysis using anti-Clox antibodies (Fig. 4). A Clox-specific band of $~190×10^3$ M_r was detected as early as in 10-day-old embryos (heads and bodies) using both α-P and α-FP antibodies. The level of this protein increased in 12- and 13-day-old embryos, where two additional specific bands of ~180 and $~250×10^3$ were also detected. The size heterogeneity of Clox proteins increased in 15-day-old embryos, where several bands, arbitrarily categorized into two groups, $~180-190×10^3$ and $~230-250×10^3$, were seen (Fig. 4B). A broad pattern of expression was observed in tissues of diverse origins, such as cartilage, liver, brain, lung, heart and skeletal muscle, with the number and relative amount of Clox-specific bands varying within tissues (Fig. 4B). Interestingly, there was a decrease of the $~180-190×10^3$ M_r Clox species in newborn (data not shown) and adult tissues (Fig. 4C), with a concomitant increase of $~230-250×10^3$ M_r Clox species. These later forms were predominant in adult brain, lung and heart whereas, in cartilage, liver and skeletal muscle, Clox proteins were barely detectable (Fig. 4C). The presence of several protease inhibitors in the homogenization buffer makes it unlikely that the predominance of smaller Clox species in embryos reflected proteolytic events. Moreover, when brains from adults and 15-day-old embryos were homogenized together, the amount of the larger Clox proteins seen in the adult tissue did not change significantly (data not shown). Thus, the mammalian *cut*-homologs are regulated during mouse development, but the diversity of protein species is higher than in *Drosophila*, where only two proteins of larger size, $~280-320×10^3$, were detected (Blochlinger et al., 1990).

The broad pattern of expression of *Clox* was confirmed by immunocytochemistry performed on 15-day-old embryos. Indeed, Clox proteins were detected in many tissues, including brain, choroid plexus, spinal cord, retina, cartilage (Figs 5, 6) and heart (Fig. 7A) and also skeletal muscle, liver, lung, thymus, tongue and several epithelia (data not shown). Preincubation of affinity-purified α-P antibodies with an excess of immunogenic peptide essentially eliminated the fluorescent signal, demonstrating its specificity (Figs 5, 6). Preincubation with equivalent amounts of an unrelated peptide did not affect either the pattern or the intensity of the signal (data not shown). Interestingly, proliferative chondrocytes showed a stronger signal than terminally differentiated hypertrophied chondrocytes (Fig. 6, compare panels A and B). Clox was also detected in the chondrogenic layer of the surrounding perichondrium, where new chondroblasts are generated (Fig. 6).

Unlike the *Antp* class homeobox genes (Shashikant et al., 1991), *Clox* did not show a gradient of expression along the anteroposterior body axis, as illustrated by the uniform signal found along the entire length of the neural axis (brain and spinal cord) in the 15-day-old embryo (Fig. 5, panels A and B, and data not shown). Instead, this pattern of expression was reminiscent of that of *cut*, which is also expressed in *Drosophila* embryos in a 'cell-type' specific rather than in a primarily 'position-specific' pattern (Blochlinger et al., 1988, 1990; Liu et al., 1991). Indeed, *cut* gene products are detected in many cells in the central nervous system, in all es organs and some peripheral neurons with multiple dendritic arborizations, and in the non-neural cells of the spiracles and Malpigian tubules. Both *Clox* and *cut* encode several protein species which, in addition to their prevalence in neural tissues, are broadly expressed during embryonic development. Whether the heterogeneity of Clox proteins reflects diversity at the mRNA level and/or post-translational modifications is presently under investigation.
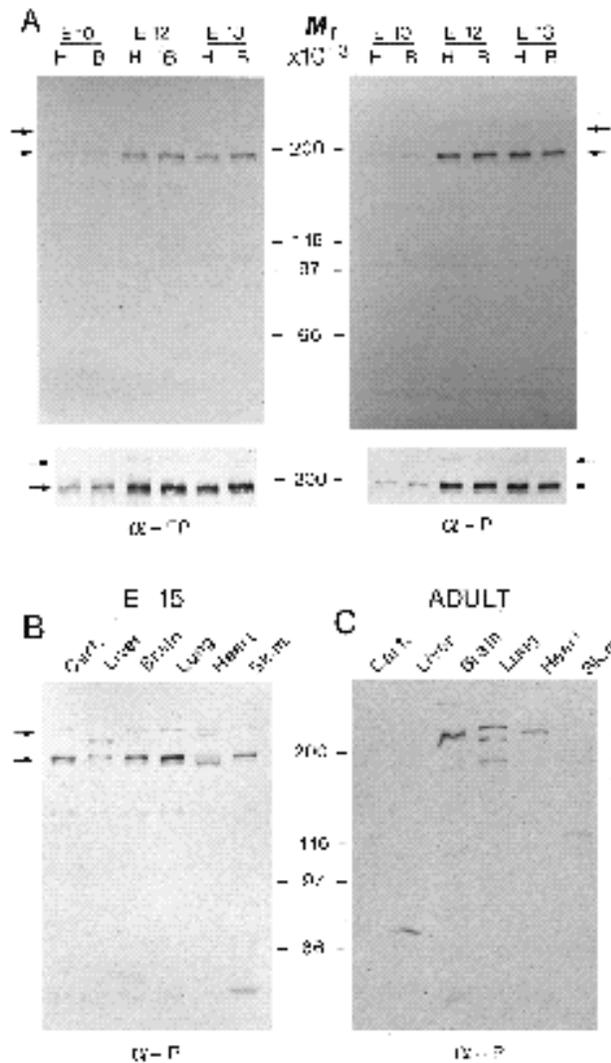
Fig. 4. Clox protein expression is developmentally regulated during mouse development. Some representative anti-Clox western immunoblots are shown. The arrows in each blot point to the two groups of Clox specific bands ($\sim$180-190$\times$10^3 M_r and $\sim$230-250$\times$10^3 M_r, see text). The faint bands below $\sim$180$\times$10^3, which were not simultaneously recognized by both antibodies (compare blots in panel A and data not shown), are not considered specific (see text). The 15-day-old cartilage extract was used as an internal control to select equivalent exposures that allowed comparison of the relative amount of protein detected in different blots. (A) Extracts from heads (H) and bodies (B) of 10-, 12- and 13-day-old embryos (E10, E12, E13) were analyzed with -FP and -P antibodies. The lower panel shows a longer exposure of the specific bands. Although not clearly visible in these reproductions, a faint band of $\sim$250$\times$10^3 was detectable in E12 and E13 extracts using both antibodies. (B and C) Tissues from 15-day-old embryos (E 15) and 1-month-old mice (ADULT) were analyzed using -P antibodies. Similar patterns were obtained with -FP antibodies (not shown). Abbreviations are: Cart., cartilage; Sk.m., skeletal muscle.

Endogenous and cloned Clox products localize to the nucleus

Determination of the subcellular localization of Clox proteins from the immunocytochemistry data shown above was complicated by the thickness of the sections and the high nuclear density in many of the tissues (see Fig. 5). However, nuclear localization was evident in tissues with lower cell density, such as heart and cartilage, where the fluorescent signal correlated with Hoechst nuclear staining (Fig. 7A). Also, the absence of signal in anuclear cells of the lens supported the conclusion that the proteins detected are nuclear (Fig. 5, panel C).

To investigate further the intracellular localization of Clox proteins, we performed indirect immunofluorescence labeling of cells in culture. The correlation between fluorescent signal and Hoechst nuclear staining found in Cos cells confirmed the subcellular localization of Clox observed in mouse tissues (Fig. 7B, a and b). This immunofluorescent pattern was specific since the signal could be largely extinguished through preincubation of -P antibodies with the immunogenic peptide (Fig. 7B, d). Other cell types tested, including myogenic Sol8 (Mulle et al., 1988), chondrogenic RCJ 3.1C5 (RCJ) (Grigordiadis et al., 1989, 1990) and HeLa cells, also showed exclusively nuclear localization of Clox (data not shown). Immunofluorescence on Cos cells transiently transfected with pMT2-Clox vectors encoding amino acids 419-975 revealed a stronger nuclear signal in 10-20% of the cells (Fig. 7B, c). This correlated well with the expected number of transfected cells overexpressing the exogenously encoded Clox protein, which was also detected as a $\sim$100$\times$10^3 band by anti-Clox western immunoblot (see below, Fig. 9B, compare lanes 8 and 9).

The above results demonstrate that, similarly to Cut, Clox proteins localize to the nucleus (see Blochlinger et al., 1988, 1990). In addition, the portion of Clox including one and a half *cut* repeat and the homeodomain is sufficient for nuclear localization (Fig. 7B, c).

Endogenous and cloned Clox products have similar DNA-binding specificity

The original Clox1 clone was identified based on its ability to bind the catenated e2 probe (see Materials and methods). However, the bacterially produced fusion proteins -gal-Clox1 and GST-Clox did not bind the monomer e2 probe (1 $\times$ probe, Fig. 8, lane 2). A minimum of two copies of the ligated probe was necessary to produce a shifted complex with GST-Clox (2 $\times$ probe, Fig. 8, lane 5). Similarly, when a Clox polypeptide (amino acids 419 to 975) was produced in rabbit reticulocyte lysate or in transfected Cos cells (see Material and methods), it showed efficient binding to Cbs-1, a synthetic direct tandem repeat of the e2 probe (Fig. 8, lanes 8 and 12, solid arrowhead). Comparable results were obtained with the protein produced from the composite Clox-cDNA, except that, as expected, a complex of slower relative mobility was observed (data not shown). The specificity of binding was demonstrated by competition with an excess of unlabeled Cbs-1 oligonucleotide, whereas an unrelated oligonucleotide of similar length did not compete binding (Fig. 8, lanes 9 and 10).

Comparison of the DNA-protein retarded complexes found in Cos cells transfected with pMT2-Clox1 expression vector and control Cos cells revealed a slow-mobility complex, which was also detected in other cell types (Fig. 8,
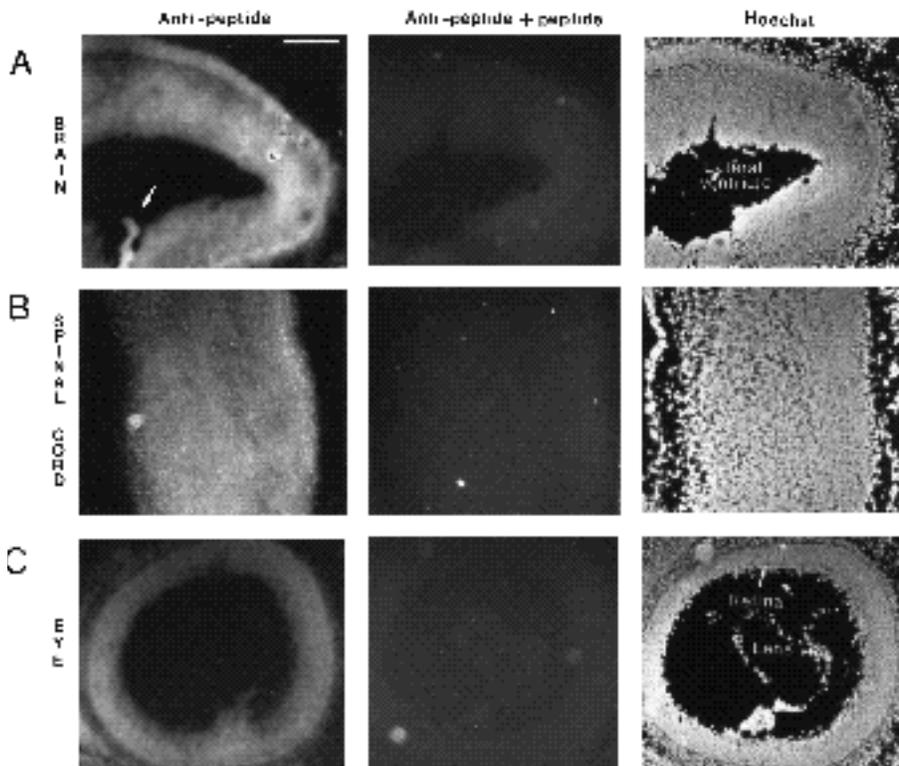
Fig. 5. Immunodetection of Clox in the 15-day-old mouse embryo. Sagittal sections were used for immunofluorescence with affinity-purified -P antibodies ("Anti-peptide"). Representative examples for the pattern observed in serial sections are shown. To test the specificity of the signal, incubations were performed using -P antibodies preincubated with 14 μM of immunogenic peptide ("Anti-peptide + peptide"). Nuclei were counterstained with Hoechst 3328 ("Hoechst"). Hoechst counterstaining corresponds to the same fields shown for the anti-peptide + peptide incubations. (Panel A) Brain (telencephalon) and choroid plexus (arrow); caudal is *down*. (Panel B) Upper part of the spinal cord. Clox was detected along the entire length of the spinal cord with no significant differences in the intensity of the signal. Dorsal is *right*, rostral is *up*. (Panel C) Section through the eye; note that Clox is detected in the retina but not in anuclear cells of the lens. Bar: 250 μm.

lanes 11 and 12; Fig. 9A, lanes 1, 3, and 6; arrow). These results were suggestive of an endogenous Clox DNA-binding activity. This was confirmed by the specific disruption of the DNA-protein complex in Sol8 myoblast extracts upon addition of -P antiserum, but not by preimmune serum (Fig. 9A, lanes 15-17). Disruption of the Clox-Cbs-1 complex was also observed upon addition of a 50-molar excess of Cbs-1 (Fig. 9A, lane 10). Other e2-containing oligonucleotides, such as Cbs-2, a 3 -5 /5 -3 repeat of the monomer e2 sequence, and Cbs-3, the −354 to −269 portion of the rat -MHC enhancer (Thompson et al., 1991), which includes one copy of the e2 sequence, also competed for binding (Fig. 9A, lanes 11 and 12). Unrelated oligonucleotides of similar size, which consisted of direct repeats of well-defined protein binding-sites, such as for MyoD1 (Weintraub et al., 1990) and T_3 receptors (Izumo and Mahdavi, 1988), did not compete for binding to Cbs-1 (Rx3 and -TRE, Fig. 9A, lanes 13 and 14).

Taken together, these experiments demonstrate that cloned and endogenous Clox proteins display very similar DNA-binding specificity. Moreover, the results indicate that specific Clox-DNA complexes were formed with ~60-mer sequences containing a minimum of one copy of the e2 monomer.

Clox DNA-binding activity and pattern of expression correlate during myogenesis and chondrogenesis in culture

As indicated above, extracts from different cell lines in culture showed Clox DNA-binding activity. Interestingly, this activity was differentiation-stage dependent in cell types that have the ability to differentiate in culture. Indeed, multipotent mesodermal C3H10T1/2 (10T1/2) cells, which can differentiate into myogenic, chondrogenic and adipogenic cells (Taylor and Jones, 1979, 1982; Konieczny and Emerson, 1984), did not bind the Cbs-1 probe (Fig. 9A, lane 2), nor did the chondrogenic RCJ cells, when maintained in nondifferentiation conditions (lane 5). In contrast, actively proliferating Sol8 myoblasts and RCJ cells in differentiation medium showed avid binding to Cbs-1 (lanes 3 and 6). Decreased binding activity was concomitant with the fully differentiated phenotype observed in nondividing Sol8 myotubes and confluent RCJ chondrocytes (lanes 4 and 7). Downregulation of binding to Cbs-1 was also observed during terminal differentiation of C2 myoblasts into myotubes (data not shown).

To correlate *Clox* pattern of expression and the observed DNA-binding activity, the cell extracts tested in the mobility shift assays were used for anti-Clox western immunoblot. Both -P (Fig. 9B) and -FP (data not shown) antibodies revealed the existence of several endogenous Clox protein species in the different cell lines. One group, with relative molecular mass of ~230-250×10^3, was present at similar levels in all cells tested irrespective of their stage of differentiation. A second group of ~180-190×10^3 M_r displayed a differentiation-stage dependent pattern of expression, which correlated with the DNA-binding activity observed in myogenic Sol8 and chondrogenic RCJ cells. Indeed, whereas the level of Clox ~180-190×10^3 was low in 10T1/2 cells (lane 2) and undifferentiated RCJ cells (lane 5), it increased significantly in Sol8 myoblasts (lane 3) and in proliferating RCJ cells maintained in differentiation medium (lane 6), but decreased again in Sol8 myotubes (lane 4) and confluent, differentiated RCJ chondrocytes (lane 7). These results are in agreement with the pattern of expression observed during mouse development, where the smaller Clox proteins were clearly expressed in differenti-
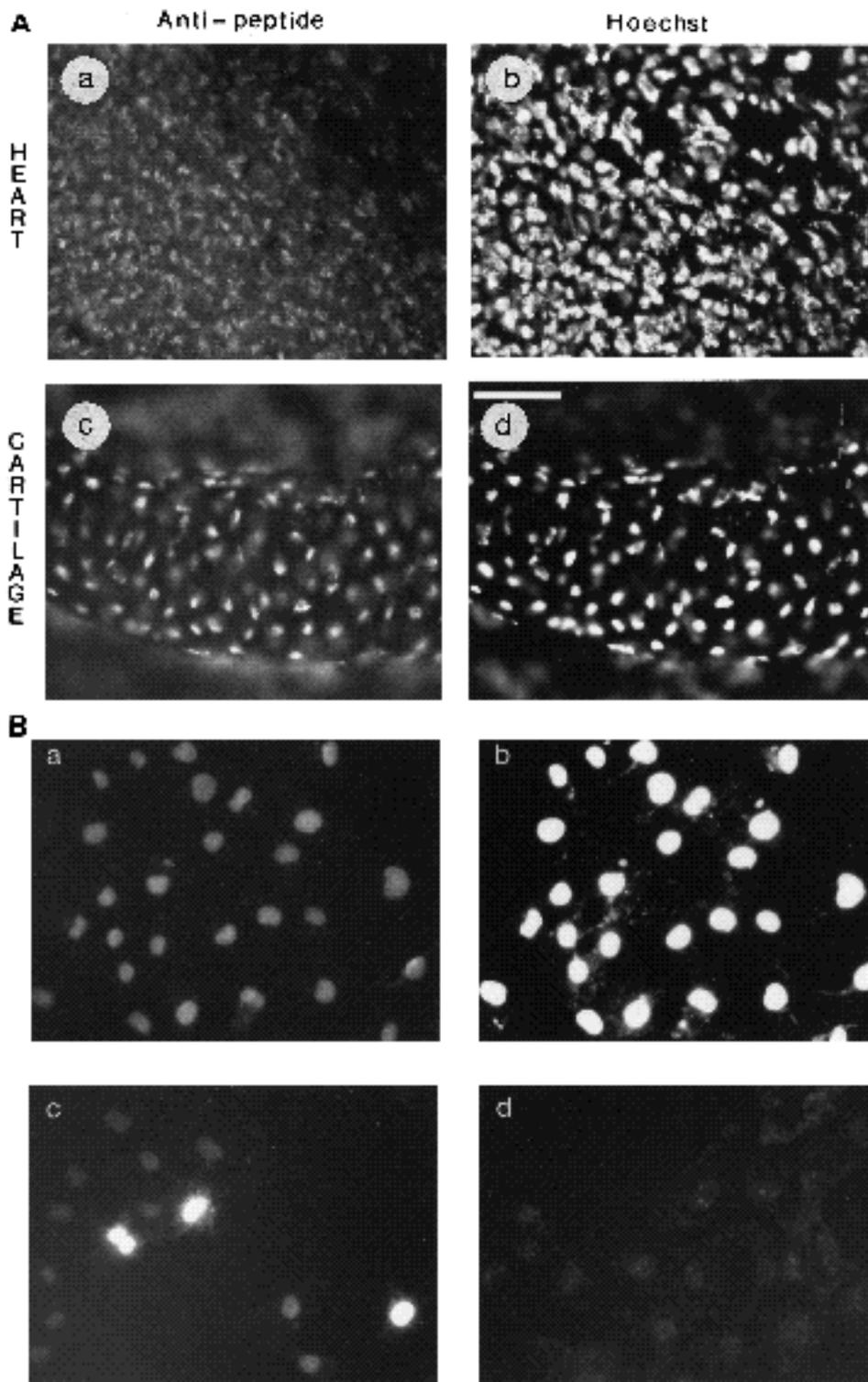
Fig. 7. Clox proteins localize to the nucleus. Immunofluorescence using affinity-purified -P antibodies and Hoechst nuclear counterstaining were performed to determine the subcellular localization of *Clox* products. (A) Sagittal sections of 15-day-old embryos, showing heart (a, b) and cartilage in the base of the skull (c, d). For details, see Fig. 5. Bar: 50 μm. (B) Immunofluorescence on Cos cells. (a) control. (b) Hoechst 33258 counterstaining of the same cells shown in a. (c) Cos cells transiently transfected with pMT2-Clox1 expression vector (amino acids 419-975). (d) as in c, except that the antibody was preincubated with 14 μM of immunogenic peptide. Cos cells transfected with pMT2 control vector showed the same pattern as control cells (not shown). The same qualitative results were obtained using protein A-purified -P antiserum and preimmune serum as control (not shown).

ating skeletal muscle and cartilage in 15-day-old embryos, but barely detected in the corresponding adult tissues (Fig. 4B, C). Additionally, as observed during the differentiation of RCJ chondrocytes in culture, immunodetection of Clox proteins in the 15-day-old mouse embryo was stronger in proliferative chondrocytes than in terminally differentiated hypertrophied chondrocytes (Fig. 6).

Thus, in various cultured cell lines, as well as in mouse embryo tissues, Clox comprises a family of ubiquitous nuclear proteins. The ~180-190×10^3 Clox subgroup, which is predominant in early embryogenesis, shows also a clear differentiation stage-specific pattern of expression during myogenesis and chondrogenesis in culture. The observation that Clox DNA-binding activity directly correlated with the
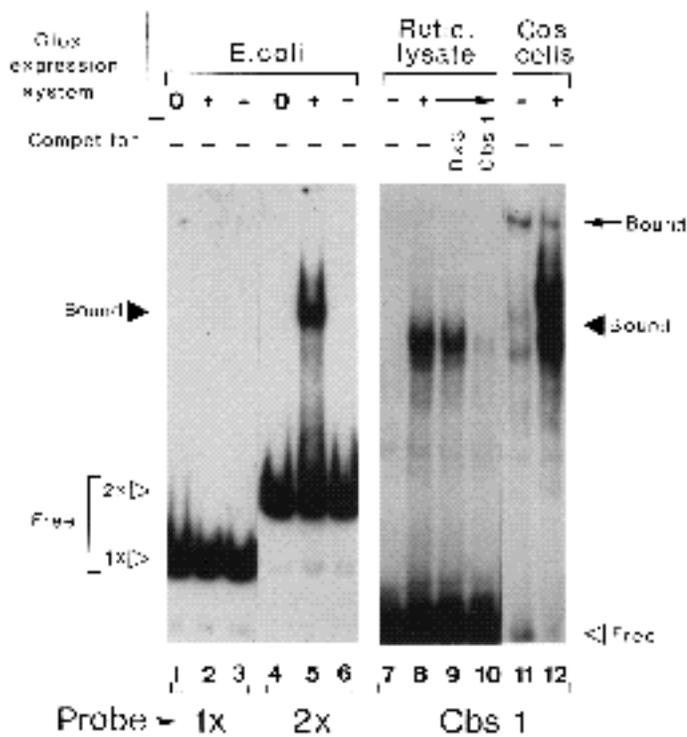
Fig. 8. Cloned Clox proteins display sequence specific DNA-binding activity. GMSA were performed using cloned Clox products produced in prokaryotic and eukaryotic expression systems. (Lanes 1-6) Bacterially produced GST-Clox fusion protein (amino acids 419-740). DNA fragments of different size from the randomly ligated e2 probe (see Materials and methods) were gel-purified and used in GMSA. Fragments corresponding to one (1 × probe) or two (2 × probe) copies were incubated with GST-Clox (+) or GST (−) (4 μg protein). No protein was added in control reactions (0). The position of free probes and bound complex are indicated (left). (Lanes 7-12) Clox1-cDNA templates (amino acids 419-975) were in vitro transcribed/translated (lanes 7-10) or transfected in Cos cells (lane 12). Lysates and extracts were incubated with Cbs-1, a direct tandem repeat of the e2 probe. Controls were, respectively, unprogrammed rabbit reticulocyte lysate (lane 7) and Cos cells transfected with pMT2 control vector (lane 11). Competition experiments (lanes 9, 10) were carried out with a 50-molar excess of unlabeled oligonucleotides; Rx3, three copies of the MyoD R-site (Weintraub et al., 1990). The indicated retarded complexes (right) correspond to expressed cloned Clox products (solid arrowhead) and endogenous Clox protein (arrow). The relative mobility of bands in lanes 1-6 and 7-12 is not comparable.

expression of the ~180-190×10³ proteins in these differentiation culture systems (compare Fig. 9A and B, lanes 1-7) suggests that the smaller protein species represent the active DNA-binding forms of Clox, at least for the target sequences used in these mobility shift assays.

Clox represses transactivation of reporter genes by muscle-specific factors

As indicated above, the original screening strategy was designed to clone a transactivator that interacts with the e2

subelement of the muscle-specific -MHC enhancer upon myogenic differentiation (Thompson et al., 1991). Since Clox is downregulated in Sol8 myotubes (Fig. 9B), it is unlikely to represent the above mentioned e2-binding factor. However, the observation that Clox interacted with oligonucleotides containing natural and artificial -MHC sequences suggested that it might be involved in the regulation of this enhancer.

To test this hypothesis, transient cotransfections were carried out in Sol8 cells using pMT2-Clox expression vectors and a CAT reporter construct bearing three copies of the e2 element, [e2]x3(rev)TK (Thompson et al., 1991). This element is sufficient, by itself, to confer muscle-specificity to a heterologous promoter (Thompson et al., 1991). To maximize the possible effect of exogenous Clox on the reporter gene, Sol8 myoblasts were switched to differentiation medium after glycerol shock (Thompson et al., 1991), so that during conversion into myotubes, endogenous Clox DNA-binding activity would be downregulated (see Fig. 9A). Cotransfection of the [e2]x3(rev)TK CAT reporter with pMT2 control vector produced the expected activation (250%) over that of the basal TK promoter, presumably due to an increase of endogenous muscle-specific e2-transactivator(s) (Fig. 10A and Thompson et al., 1991). Cotransfection with pMT2-Clox-sense strongly reduced the activity of [e2]x3(rev)TK CAT, by 80%, with no significant effect on the basal promoter activity (TK CAT). These results suggested that Clox prevented transactivation of the reporter construct by blocking the interaction of the muscle-specific factors with the e2 element. Alternatively, the negative transcriptional regulatory activity of Clox might be due to the absence of its amino-terminal coding portion in the expression construct. The latter possibility was ruled out in assays using pMT2-Clox-antisense, which produced an additional (180%) activation of [e2]x3(rev)TK CAT, again without affecting the basal activity of the promoter (TK CAT). This effect could be attributed to a further reduction of endogenous Clox, resulting from the hybridization of endogenous sense and transfected antisense *Clox* transcripts, thereby titrating out the inhibitory effect of Clox while facilitating the interaction of positive factors.

We have recently observed that MEF2, a muscle-specific transcription factor cloned in our laboratory (Yu et al., 1992), transactivated the expression of the -MHC enhancer in the nonmuscle CV1 cells (Mahdavi, unpublished data). Cotransfections were then performed to test the effect of Clox on MEF2-mediated transactivation of the natural -MHC enhancer sequences. The MEF-2 expression plasmid pMT2-MEF2 produced a 5000% activation on the -MHC enhancer CAT construct, [−285/−180] A10, with no effect on the basal promoter activity (Fig. 10B, lanes 1, 2, 5 and 6). As expected, pMT2-Clox-sense reduced by 40% the MEF2-mediated transactivation of the -MHC reporter (Fig. 10B, compare lanes 2 and 3). When pMT2-MEF2 and pMT2-Clox-antisense were cotransfected, 6000% activation of the reporter was observed instead (Fig. 10B, lane 4). These data clearly demonstrate the inhibitory function of Clox, which, by interacting with the -MHC enhancer sequences, appears to interfere with MEF2 or MEF2-regulated transactivators. Taken together, these data are consis-
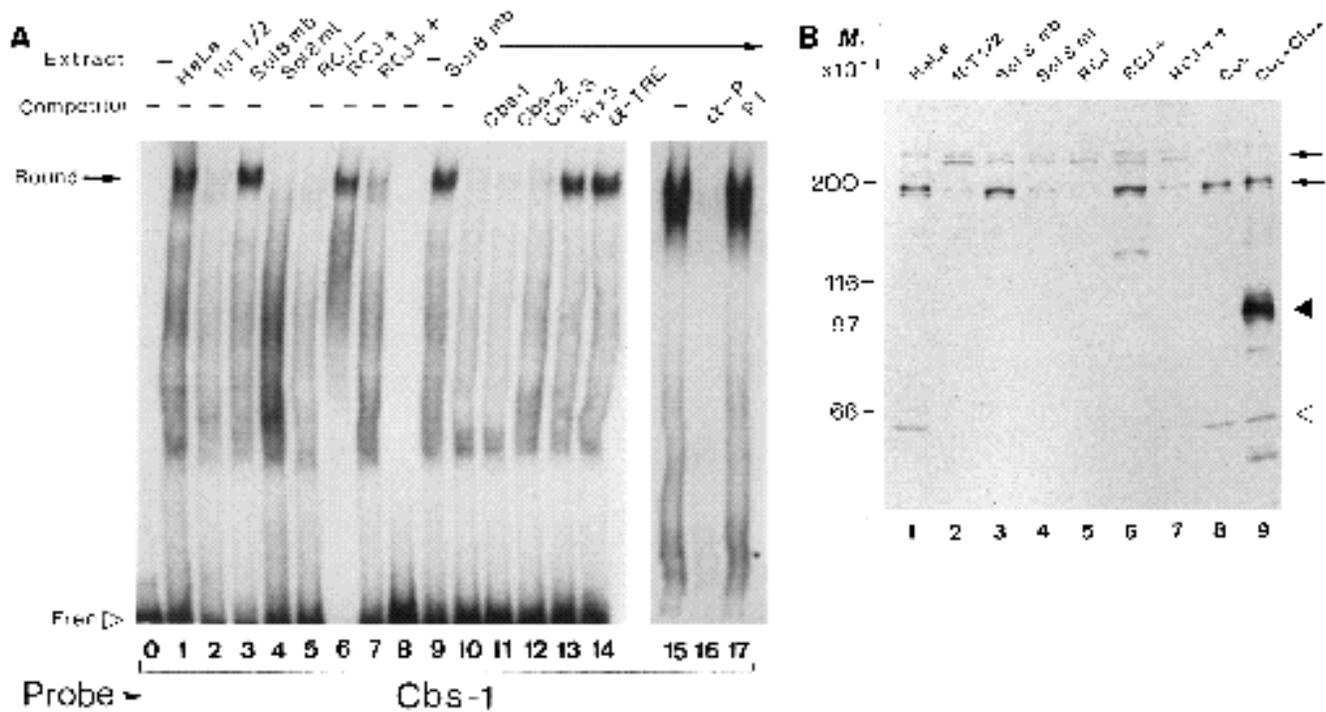
Fig. 9. Clox displays differentiation stage-specific DNA-binding activity and expression pattern in cells in culture. (A) GMSA using Cbs-1 probe and extracts from different cell lines in culture. Sol8 mb, myoblasts; Sol8 mt, myotubes; RCJ cells were maintained either in standard medium (−) or differentiation medium (RCJ+, 4 days; RCJ++, 8 days) (see Materials and methods). (Lanes 9-14) Competition with a 50 molar excess of unlabeled oligonucleotides. Cbs-2 and Cbs-3, see Materials and methods; Rx3, three copies of the MyoD R-site (Weintraub et al., 1990); α-TRE, thyroid responsive element of the rat α-MHC gene (−161 to −124 sequence; Izumo and Mahdavi, 1988). (Lanes 15-17) Effect of α-P antiserum (α-P) or preimmune serum (PI) on Clox/Cbs-1 retarded complex in Sol8 myoblast extracts. A longer run than that shown in lanes 0-14 was performed to resolve possible supershifted complexes. (B) Western blot analysis using α-P antibodies and the same cell extracts tested in the GMSA shown in A. Cos-Clox refers to Cos cells transiently transfected with pMT2-Clox1 expression vector (amino acids 419-975); the exogenously expressed Clox protein is indicated (solid arrowhead). The arrows point to the two groups of Clox specific bands (~180-190×10^3 and ~230-250×10^3, see text). Although not clearly visible in this reproduction, the high molecular weight Clox proteins were detected in all cell extracts. Similar pattern was observed using α-FP antibodies. No proteins were detected after preincubation of α-P antibodies with 12 µM of immunogenic peptide, except for the ~60×10^3 band in HeLa and Cos cells (open arrowhead). Similar DNA-binding activity and pattern of expression were observed with different cell extracts.

tent with the role of Clox as a developmentally regulated repressor of tissue-specific gene transcription.

Discussion

The evolutionary conservation of the homeodomain and *cut* repeats found in the *Drosophila cut* gene product (Blochlinger et al., 1988), and in Clox, its mammalian counterpart described here, is striking. Indeed, with the exceptions of the POU and paired domains of some homeoproteins (Shashikant et al., 1991), similarities between related homeoproteins are mainly limited to the homeodomain and immediate flanking regions. Thus, the conservation of the *cut* repeats suggests that this ~73 amino acid motif is likely to represent a new functional domain, which may define a family of *cut*-homeodomain proteins. It has been suggested that the *cut* repeats have arisen recently in insect evolution by duplication (Blochlinger et al., 1988), but their conservation in Clox indicates that the duplication events must have indeed predated the divergence between insects and mammals. We show here that other common features

between *Clox* and *cut* gene products include the existence of diverse protein species in several tissues and regulation of their expression during embryogenesis. We also show that *Clox* levels of expression and DNA-binding activity, which are differentiation stage-specific, are highest in cells committed to a differentiation pathway, such as chondroblasts and myoblasts, but drop dramatically when these cells have reached their fully differentiated phenotype. These observations, together with the evidence that the cloned Clox isoform, which is a nuclear DNA-binding protein, acts as a transcriptional inhibitor, are consistent with the hypothesis that *cut*-homeoproteins are modulators of stage-specific gene expression.

The diversity of Clox and Cut proteins can be generated by a combination of alternative splicing events and post-translational modifications. Blochlinger et al. (1988) have detected at least four different *cut* transcripts, although they could not find any evidence for alternative splicing within the coding sequences (Blochlinger et al., 1990). In contrast, Liu et al. (1991) have suggested that different Cut tissue-specific isoforms might be generated by alternative splicing. The heterogeneity of Clox transcripts observed in sev-

A

Sol 8

pMT2-Clox [Sense	—	2	10	—	—	—	2	10	—	—
Antisense	—	—	—	2	10	—	—	—	2	10
pMT2	15	13	5	13	5	15	13	5	13	5
Reporter	[βe2] x3 (rev) TK CAT					TK CAT				

B

CV1

	1	2	3	4	5	6	7	8
pMT2-MEF2	—	5	5	5	—	5	5	5
pMT2-Clox [Sense	—	—	5	—	—	—	5	—
Antisense	—	—	—	5	—	—	—	5
pMT2	10	5	—	—	10	5	—	—
Reporter	[-285/-180] A10 CAT				A10 CAT			
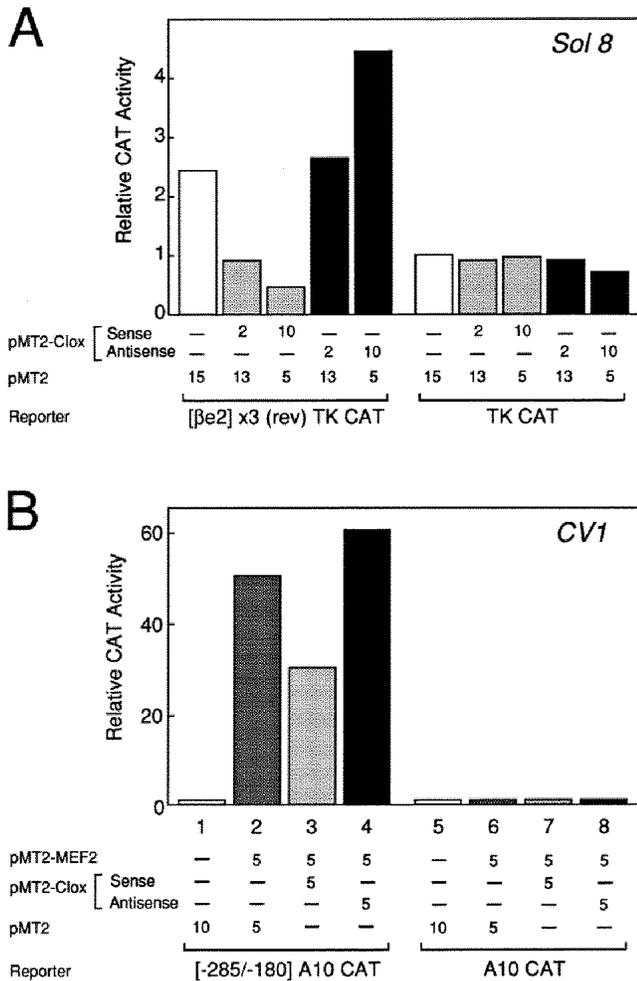
Fig. 10. Clox inhibits muscle-specific gene transactivation. Cells were transiently cotransfected with pMT2 expression vectors and chloramphenicol acetyl-transferase (CAT) reporter constructs, along with MLV β-galactosidase plasmid to control for transfection efficiency. Similar results (s.d. < 20%) were obtained in three independent experiments with different plasmid preparations. See text and Materials and methods for details. (A) Sol8 myoblasts were cotransfected with the indicated amounts (μg) of pMT2-Clox1 expression vector (sense or antisense) or control pMT2, and 5 μg of CAT reporter constructs under the control of either the basal HSTK promoter (TK CAT), or including three copies of the e2 subelement of the rat β-MHC enhancer ([βe2]x3(rev) TK CAT) (Thompson et al., 1991). (B) CV1 cells were cotransfected with the indicated amounts (μg) of pMT2 expression vectors or control pMT2, and 10 μg of CAT reporter constructs under the control of either the basal SV40 promoter (A10 CAT), or including the sequence from −285 to −180 of the rat β-MHC enhancer ([−285/−180] A10 CAT) (Thompson et al., 1991). Activities are expressed in -fold increase over the activity in uninduced cells (= 1, lane 1).

eral tissues (data not shown) and the similarity of Clox and CDP (Neufeld et al., 1992) sequences provide evidence that a number of Clox variants are produced, in part, by alternative splicing of the same gene product.

Role of Clox as modulators of gene expression

All available evidence suggests that *cut* products are transcriptional regulators, although Cut DNA-binding activity, or putative target genes have not yet been reported. We show here that cloned and endogenous Clox proteins from different cultured cell lines exhibit similar DNA-binding characteristics and specificity. GST-Clox (amino acids 419-740) had DNA-binding activity, indicating that a portion of Clox, starting at the second *cut* repeat and including the homeodomain, is sufficient for this activity. In addition, both bacterially made GST-Clox and in vitro translated Clox products bound DNA, suggesting that neither heterodimerization nor other cellular factors are required for this activity. However, we can not exclude the possibility that interactions with other cellular factors might be involved in the regulation of the binding activity observed in cell extracts.

During myogenesis, upon terminal differentiation of myoblasts into myotubes, downregulation of *Clox* expression and DNA-binding activity are concomitant with the developmentally regulated transactivation of the β-MHC gene (the results here and Thompson et al., 1991). These observations, together with the repressor activity of Clox on β-MHC expression in cotransfection assays, suggest that Clox functions as an inhibitor of gene transcription by preventing the interaction of tissue-specific transactivators with their cognate target sequences. As indicated above, a human counterpart of *cut* has been cloned independently by others (Neufeld et al., 1992). Based on its DNA-binding characteristics, Neufeld and colleagues have proposed that the human *cut* homolog corresponds to the previously reported CCAAT-displacement protein (CDP) (Barberis et al., 1987; Superti-Furga et al., 1988, 1989; Skalnik et al., 1991). CDP has been previously shown to bind the upstream regulatory sequences of diverse tissue-specific genes, such as the cytochrome b heavy chain (gp91-phox) (Skalnik et al., 1991), the sea urchin sperm histone H2B (Barberis et al., 1987) and the human β-globin (Superti-Furga et al., 1988, 1989). Interestingly, downregulation of CDP DNA-binding activity coincided with the induction of expression of these genes upon differentiation of the expressing cell types, suggesting that CDP may be an inhibitor of gene transcription. In addition, the predicted size of the CDP (Superti-Furga et al., 1989; Skalnik et al., 1991; Neufeld et al., 1992) was similar to that of the Clox DNA-binding forms.

Despite the apparent similarities between Clox and CDP, comparison of CDP footprinted regions and Clox-binding sites did not reveal a consensus sequence. Specifically, Cbs-1 has no CCAAT motifs, which are characteristic of CDP target genes (Barberis et al., 1987; Superti-Furga et al., 1988; Skalnik et al., 1991). These observations suggest that, if Clox and CDP are related, their DNA-binding specificity differ in diverse regulatory contexts, or they recognize a variety of apparently unrelated sequences. In addition, Clox expressed from the cloned cDNA did not bind the gp91-phox promoter sequence bound by the human *cut* homolog (data not shown). Because our clone lacks the amino terminus, we cannot rule out the involvement of these sequences in the determination of binding specificity. How-

ever, the fact that cloned and endogenous Clox proteins have very similar binding characteristics make this possibility unlikely. Instead, the different carboxy terminal portions of the cloned Clox and human *cut* homolog might account for different target gene specificity. The characterization of additional Clox variants, now in progress, should elucidate these points.

Clox as putative regulators of diverse differentiation programs

Several lines of evidence indicate that *cut* establishes tissue identity by specifying diverse programs of differentiation in precursor cells (Bodmer et al., 1987; Blochlinger et al., 1988, 1990, 1991; Liu et al., 1991). The developmental regulation of *Clox* expression in mouse embryonic tissues suggests that *Clox* might be also involved in cell type specification and morphogenesis in diverse differentiation programs. This is consistent with the patterns of Clox expression and DNA-binding activity in cells in culture, which are upregulated during commitment of precursor cells to a differentiation pathway, and downregulated later during terminal differentiation.

Unlike the *Antp* class homeotic genes, both *Clox* and *cut* exhibit tissue-specific rather than region-specific expression pattern. *Hlx*, a murine homeobox gene which also belongs to the divergent homeoprotein class (Shashikant et al., 1991), is expressed in diverse embryonic and adult tissues (Allen et al., 1991). The existence of these potentially 'multifunctional' homeobox genes raises an important question as to how they might function in such divergent differentiation programs. The apparent diversity of Cut and Clox protein species is intriguing, and may reflect functional differences. This might be achieved by regulated alternative splicing (reviewed in Smith et al., 1989). Indeed, alternative splicing of I-POU transcripts generates an activator as well as an inhibitor of gene transcription, which modulate two distinct regulatory programs during neural development (Treacy et al., 1992). Also, alternative splicing of Oct-2 transcripts can change promoter selectivity, without affecting DNA-binding specificity (Tanaka et al., 1992). It is also noteworthy to point out that both *Clox* and *cut* homeodomains have Asn at position 47, which has been suggested to participate in regulating DNA-binding activity by promoting homo- and heterodimerization (Rayle, 1991). Thus, association with distinct stage-specific and/or tissue-specific cofactors might also confer specificity to *Clox* function.

A network of upstream and downstream genes would add an additional level of complexity to the regulation of gene expression via these 'multifunctional' transcriptional factors. Particularly interesting would be to ascertain whether this network is similar in different cell lineages, or, alternatively, there is cell-type specificity. Determination of neural precursor cells by the *Drosophila* proneural genes *daughterless* (*da*) and the *achaete-scute* complex (*AS-C*) seems to occur prior to *cut* function (for reviews, see Ghysen and Dambly-Chaudiere, 1989; Campos-Ortega and Knust, 1990; Jan and Jan, 1990). Subsequently, *cut* specifies the individual identities of the neural precursors defined by the former regulators (Blochlinger et al., 1991). The regulated expression of Clox proteins in diverse mouse neural

tissues, such as brain, spinal cord, neural retina and choroid plexus, suggests that *Clox* may play a similar role in the development of the mammalian nervous system. Because both *da* and *AS-C* gene products are basic-helix-loop-helix (bHLH) proteins (Campos-Ortega and Knust, 1990), it is tempting to postulate that lineage-specific bHLH proteins might regulate *Clox* and *cut* expression in different committed precursor cells. For instance, MASH-1 and/or MASH-2, the bHLH mammalian counterparts of *AS-C* (Johnson et al., 1990; Lo et al., 1991), might regulate *Clox* expression in the nervous system. Similarly, myogenic bHLH transactivators of the MyoD1 gene family (reviewed in Olson, 1990; Weintraub et al., 1991) might determine *Clox* expression in skeletal muscle, consistent with Clox upregulation in committed myoblasts (Fig. 9B). This network could be further expanded by auto and/or crossregulatory interactions between homeoproteins, competence for binding to similar *cis* regulatory sequences, posttranslational modifications and interaction with other cellular factors.

References

Allen, J. D., Lints, T., Jenkins, N. A., Copeland, N. G., Strasser, A., Harvey,R.P.andAdams,J.M. (1991). Novel murine homeobox gene on chromosome 1 is expressed in specific hematopoietic lineages and during embryogenesis. *Genes Dev.* **5**, 509-520.

Arcioni, L.,Simeone, A., Guazzi, S., Zappavigna, V., Boncinelli, E. and Mavilio, F. (1992). The upstream region of the human homeobox gene HOX3D is a target for regulation by retinoic acid and HOX homeoproteins. *EMBO J.* **11**, 265-277.

Barberis, A., Superti-Furga, G. and Busslinger, M. (1987). Mutually exclusive interaction of the CCAAT-binding factor and of a displacement protein with overlapping sequences of a histone gene promoter. *Cell* **50**, 347-359.

Blochlinger, K., Bodmer, R., Jack, J. W., Jan, L. Y. and Jan, Y. N. (1988). Primary structure and expression of a product from *cut*, a locus involved in specifying sensory organ identity in *Drosophila.Nature* **333**, 629-635.

Blochlinger,K.,Bodmer,R., Jan, L. Y. and Jan, Y. N. (1990). Patterns of expression of Cut, a protein required for external sensory organ development in wild-type and *cut* mutant *Drosophila* embryos. *Genes Dev.* **4**, 1322-1331.

Blochlinger, K., Jan, L. Y. and Jan, Y. N. (1991). Transformation of sensory organ identity by ectopic expression of Cut in *Drosophila*. *Genes Dev.* **5**, 1124-1135.

Bodmer,R.,Barbel, S., Sheperd, S., Jack, J. W., Jan, L. Y. and Jan, Y. N. (1987). Transformation of sensory organs by mutations of the *cut* locus of *D. melanogaster. Cell* **51**, 293-307.

Campos-Ortega, J. A. and Knust, E. (1990). Genetics of early neurogenesis in *Drosophila melanogaster. Ann. Rev. Genet.* **24**, 387-407.

Ghysen, A. and Dambly-Chaudiere, C. (1989). Genesis of the *Drosophila* peripheral nervous system. *Trends Genet.* **5**, 251-255.

Grigoriadis, A.E., Aubin, J. E. and Heersche, J. N. M. (1989). Effects of dexamethasone and vitamine D₃ on cartilage differentiation in a clonal chondrogenic cell population. *Endocrinology* **125**, 2103-2110.

Grigoriadis, A. E., Heersche, J. N. M. and Aubin, J. E. (1990). Continously growing bipotential and monopotential myogenic, adipogenic, and chondrogenic subclones isolated from the multipotential RCJ 3.1 clonal line. *Dev. Biol.* **142**, 313-318.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Hayashi, S. and Scott, M. P. (1990). What determines the specificity of action of *Drosophila* homeodomain proteins? *Cell* **63**, 883-894.

Holland, P.W.H. and Hogan, B. L. M. (1988). Expression of homeo box genes during mouse development: a review. *Genes Dev.* **2**, 773-782.

Hopp, T. P. and Woods, K. R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* **78**, 3824-3838.

Izumo, S. and Mahdavi, V. (1988). Thyroid hormone receptor isoforms generated by alternative splicing differentially activate myosin HC gene transcription. *Nature* **334**, 593-595.

Jack, J. W. (1985). Molecular organization of the *cut* locus of *Drosophila melanogaster. Cell* **42**, 869-876.

Jan, L.Y. and Jan, Y. N. (1990). Genes required for specifying cell fates in *Drosophila* embryonic sensory nervous system. *Trends Neurosci.* **13**, 494-498.

Johnson, T. K. and Judd, B. H. (1979). Analysis of the *cut* locus of *Drosophila melanogaster. Genetics* **92**, 485-502.

Johnson, J. E., Birren, S. J. and Anderson, D. J. (1990). Two rat homologues of *Drosophila achaete-scute* specifically expressed in neural precursors. *Nature* **346**, 858-861.

Kaufman, R. J., Davies, M. W., Pathak, V. K. and Hershey, J. W. B. (1989). The phosphorylation state of eukaryotic initiation factor 2 alters translational efficiency of specific mRNAs. *Mol. Cell. Biol.* **9**, 946-958.

Konieczny, S.F. and Emerson,C. P., Jr. (1984). 5-azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. *Cell* **38**, 791-800.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Liu, S., McLeod, E. and Jack, J. (1991). Four distinct regulatory regions of the *cut* locus and their effect on cell type specification in *Drosophila. Genetics* **127**, 151-159.

Lo, L., Johnson, J. E., Wuenschell, C. W., Saito, T. and Anderson, D. J. (1991). Mammalian *achaete-scute* homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. *Genes Dev.* **5**, 1524-1537.

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.

Mulle, C., Benoit, P., Pinset, C., Roa, M. and Changeux, J.-P. (1988). Calcitonin gene-related peptide enhances the rate of desenzitation of the nicotinic acetylcholine receptor in cultured mouse muscle cells. *Proc. Natl. Acad. Sci. USA* **85**, 5728-5732.

Neufeld,E.J.,Skalnick, D. G., Lievens, P. M.-J. and Orkin, S. H. (1992). Human CCAAT displacement protein is homologous to the *Drosophila* homeoprotein, *cut. Nature Genetics* **1**, 50-55.

Olson, E. N. (1990). MyoD family: a paradigm for development? *Genes Dev.* **4**, 1454-1461.

Rayle, R. E. (1991). The oncofetal gene *Pem* specifies a divergent paired class homeodomain. *Dev. Biol.* **146**, 255-277.

Reznikoff, C. A., Brankow, D. W. and Heidelberger, C. (1973). Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.* **33**, 3231-3238.

Rosenfeld, M. G. (1991). POU-domain transcription factors: pou-er-ful developmental regulators. *Genes Dev.* **5**, 897-907.

Ruvkun, G. and Finney, M. (1991). Regulation of transcription and cell identity by POU domain proteins. *Cell* **64**, 475-478.

Sambrook, J.,Fritsch,E. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schneuwly, S., Kuroiwa, A., Baumgartner, P. and Gehring, W. J. (1986). Structural organization and sequence of the homeotic gene *Antennapedia* of *Drosophila melanogaster. EMBO J.* **5**, 733-739.

Schöler, H.R. (1991). Octamania: the POU factors in murine development. *Trends Gen.* **7**, 323-329.

Scott, B., Simmerman, H., Collins, J., Nadal-Ginard, B. and Jones, L. (1988). Complete amino acid sequence of canine cardiac calsequestrin deduced by cDNA cloning. *J. Biol. Chem.* **263**, 8958-8964.

Scott, M. P., Tamkun, J. W. and Hartzell, G. W., III. (1989). The structure and function of the homeodomain. *Biochim. Biophys. Acta* **989**, 25-48.

Shashikant, C. S., Utset, M. F., Violette, S. M., Wise, T. L., Einat, P., Pendleton, J. W., Schugart, K. and Ruddle, F. H. (1991). Homeobox genes in mouse development. *Critical Reviews in Eukaryotic Gene Expression* **1**, 207-245.

Singh, H., LeBowitz, J. H., Baldwin, A. S., Jr. and Sharp, P. A. (1988). Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. *Cell* **52**, 415-423.

Skalnik, D. G., Strauss, E. C. and Orkin, S. H. (1991). CCAAT displacement protein as a repressor of the myelomonocytic-specific gp91-phox gene promoter. *J. Biol. Chem.* **266**, 16736-16744.

Smith, D. B. and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31-40.

Smith, C.W.J., Patton, J. G. and Nadal-Ginard, B. (1989). Alternative splicing in the control of gene expression. *Annu. Rev. Genet.* **23**, 527-577.

Superti-Furga, G., Barberis, A., Schaffner, G. and Busslinger, M. (1988). The −117 mutation in greek HPFH affects the binding of the three nuclear factors to the CCAAT region of the -globin gene. *EMBO J.* **7**, 3099-3107.

Superti-Furga, G., Barberis, A., Schreiber, E. and Busslinger, M. (1989). The protein CDP, but not CP1, footprints on the CCAAT region of the -globin gene in unfractionated B-cell extracts. *Biochim. Biophys. Acta* **1007**, 237-242.

Tanaka, M., Lai, J. and Herr, W. (1992). Promoter-selective activation domains in Oct-1 and Oct-2 direct differential activation of an snRNA and mRNA promoter. *Cell* **68**, 755-767.

Taylor, S.M., and Jones, P. A. (1979). Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell* **17**, 771-779.

Taylor, S.M., and Jones, P. A. (1982). Changes in phenotypic expression in embryonic and adult cells treated with 5-azacytidine. *J. Cell Physiol.* **111**, 187-194.

Thompson, W.R., Nadal-Ginard, B. and Mahdavi, V. (1991). A MyoD1-independent muscle-specific enhancer controls the expression of the -myosin heavy chain gene in skeletal and cardiac muscle cells. *J. Biol. Chem.* **266**, 22678-22688.

Treacy, M.N., Neilson, L. I., Turner, E. E., He, X. and Rosenfeld, M. G. (1992). Twin of I-POU: a two amino acid difference in the I-POU homeodomain distinguishes and activator from an inhibitor of transcription. *Cell* **68**, 491-508.

Weintraub, H., Davis, R., Lockshon, D. and Lassar, A. (1990). MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. *Proc. Natl. Acad. Sci. USA* **87**, 5623-5627.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y. and Lassar, A. (1991). The MyoD gene family: nodal point during specification of the muscle cell lineage. *Science* **251**, 761-765.

Young, R. A. and Davies, R. W. (1983). Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA* **80**, 1194-1198.

Yu, Y.-Y., Breitbart, R. E., Mahdavi, V. and Nadal-Ginard, B. (1992).

Human myocite-specific enhancer factor 2 (MEF2) comprises a group of transcription factors belonging to the MADS gene family. *Genes Dev.* **6**, 1783-1798

Zappavigna, V., Renucci, A., Izpisúa-Belmonte, J.-C., Urier, G., Peschle, C. and Duboule, D. (1991). HOX4 genes encode transcription factors with potential auto- and cross-regulatory capacities.*EMBO J.* **10**, 4177-4187.

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Fig. 6. Immunodetection of Clox in cartilaginous structures at different stages of chondrogenesis in the 15-day-old mouse embryo. For routine histology, cresyl violet was used, which stains the extracellular matrix of cartilage (pink staining). (Panel A) Proliferating chondrocytes in costal cartilage, which connects the rib to the sternum. (Panel B) Hypertrophied degenerating chondrocytes in the cartilage precursor of the clavicle. Note that the stronger fluorescent signal was detected in the inner chondrogenic layer of the perichondrium (arrowhead) and in proliferative chondrocytes. Bar: 100 µm.