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

VICENTE ANDRES, BERNARDO NADAL-GINARD and VIJAK MAHDAVI

Howard Hughes Medical Institute and Department of Cardiology, Children’s Hospital, Department of Cellular and Molecular Physiology and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

1Author for correspondence

**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, ~180-190×10³ M_r Clox proteins predomi-

**Introduction**

Molecular and genetic analyses have demonstrated that homebox-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 homoeoprotein 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 homoeoproteins. 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 passaged 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 (α-Minimum Essential Medium + 15% fetal bovine serum) or differentiation medium (standard medium supplemented with 10 mM sodium β-glycerophosphate, 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). RCJ 3.1C5 (RCJ) chondrogenic cells were maintained either in standard medium (α-Minimum Essential Medium + 15% fetal bovine serum) or differentiation medium (standard medium supplemented with 10 mM sodium β-glycerophosphate, 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 Xgt11 cDNA library from adult dog cardiac ventricle (Scott et al., 1988) was screened using a randomly ligated DNA probe consisting of the β2 subelement from the rat β-MHC gene 5′-upstream sequences, position −288 to −267 (Thompson et al., 1991), along with cohesive BgIII sites. To prepare the catedenated probe, the ends of the monomer fragment were filled in with [α-32P]dATP, dCTP, dGTP and dTTP using Klenow enzyme, and the resulting 31 base pair blunt-ended DNA fragment, 5′-GATCT-GTGAAGCTTGAATGAGGAGATC-3′ (denoted β2 or 1× probe), was ligated with T4 DNA ligase. Lytic growth of bacteriophage Xgt11 in E.coli Y1090 and transfer of β-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 catedenated probe were carried out essentially as described by Singh et al. (1988). In a screen of 5 × 106 recombinants, one positive phage, designated AClx1, was isolated. Screening of the corresponding Xgt10 library with AClx1-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 EcoRI/BglII fragment including nucleotides 1-1130 to a BglII/KpnI fragment containing nucleotides 1131-3379 (excised from pGEM3-Clox2 and pGEM3-Clox3, respectively; see Fig. 2A). The resulting EcoRI/KpnI 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 EcoRI 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, [β2]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 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) β-galactosidase plasmid to control for transfection efficiency.

Antiseras 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 antipeptide (α-P) serum was further affinity purified by passing 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 BamHI-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 (GlutageneTM). The fusion protein was overexpressed in E. coli (DH5α) and purified essentially as described by Smith and Johnson (1988). To raise the α-FP antiserum, a gel slice containing the fusion protein (~73×10⁵ M0) 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 (−20°C), 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 α-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 (1:600, Cappel), and sections were fixed for 10 minutes in PBS. The sections 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) and blue light microscopy 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) and blue light microscopy were stained with cresyl violet for routine histology.

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 4°C 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 4°C 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 2x 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 4°C. 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 semidy 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 37°C with 25 mM Tris-Cl (pH 8.0), 125 mM NaCl, 0.05% Tween-20 (TBST), containing 4% nonfat dry milk. Incubations with α-P and α-FP antibodies diluted to 4-7 µg/ml with TBST, 2% nonfat milk were performed for 8-12 hours at 4°C. Washes, incubations with secondary antibody and visualization of the immune complexes were carried out as recommended by the manufacturer (Enhanced Chemiluminescence 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 α-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 4°C 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 β2 probe (see above) were synthesized and named Chs-1 (5′-3′/5′-3′) and Chs-2 (3′-5′/5′-3′). Chs-3 consisted of the sequence from −354 to −269 of the rat β-MHC enhancer, which contains a single copy of the β2 element (Thompson et al., 1991). Oligonucleotides were end labeled using T4 kinase and [γ-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 polyclonal antibody (diluted 1:100 in gel) before being loaded onto 6.5% SDS-polyacrylamide gels. The ~3.4 kb Clox cDNA sequence predicted an open reading frame which encodes a polypeptide of 975 amino acids at a poly(A) tract. This open reading frame encodes a polypeptide 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 homeo-protein (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 Glu→Glu substitution at position 12, which has been also changed to Ala.

**Results**

**Isolation of Clox cDNAs and predicted protein sequence**

The λClox1 cDNA was initially isolated from a dog heart λgt11 expression library, in an attempt to clone a cardiac transcription factor that interacts with the β2 subelement of the sarcomeric β-myosin heavy chain (β-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 λClox1 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 the in-frame TGA stop codon (nucleotides 2926-2928), and the in-frame Gln→Glu subelement at position 12, which has been also changed to Ala.
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 α-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).
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 (α-P) specifically recognized both the immuno- genic peptide and GST-Clox fusion protein, but not GST. Anti-GST-Clox antibodies (α-FP) only recognized GST-Clox, although this peptide sequence is contained in GST-Clox. These results were confirmed by ELISA (data not shown). α-P antibodies immunoprecipitated in vitro translated Clox products (Fig. 3B). Immunoprecipitated products were not detected when the affinity-purified α-P antibody was preincubated with immunogen 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-
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 Mr 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 Mr Clox species in newborn (data not shown) and adult tissues (Fig. 4C), with a concomitant increase of ~230-250×10^3 Mr 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 Malpighian 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.
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 α-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 ~100×10^3 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 λClox1 clone was identified based on its ability to bind the catenated βe2 probe (see Materials and methods). However, the bacterially produced fusion proteins β-gal-Clox1 and GST-Clox did not bind the monomer βe2 probe (1 × 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 × 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 βe2 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, lane 9).
 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 α-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 βe2-containing oligonucleotides, such as Cbs-2, a 3′-5′/5′-3′ repeat of the monomer βe2 sequence, and Cbs-3, the –354 to –269 portion of the rat β-MHC enhancer (Thompson et al., 1991), which includes one copy of the βe2 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 T3 receptors (Izumo and Mahdavi, 1988), did not compete for binding to Cbs-1 (Rx3 and α-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 βe2 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 immunofluorescence with affinity-purified α-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 α-P antibodies preincubated with 14 μM of immunogen 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.
Clox, a mammalian cut homolog

329

Clox, a mammalian cut homolog, is expressed in 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. 7. Clox proteins localize to the nucleus. Immunofluorescence using affinity-purified α-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 α-P antiserum and preimmune serum as control (not shown).
expression of the ~180-190×10^3 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 βe2 subelement of the muscle-specific β-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 βe2-binding factor. However, the observation that Clox interacted with oligonucleotides containing natural and artificial β-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 βe2 element, [βe2]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 [βe2]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 βe2-transactivator(s) (Fig. 10A and Thompson et al., 1991). Cotransfection with pMT2-Clox-sense strongly reduced the activity of [βe2]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 βe2 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 [βe2]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 β-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 β-MHC enhancer sequences. The MEF-2 expression plasmid pMT2-MEF2 produced a 5000% activation on the β-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 β-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 β-MHC enhancer sequences, appears to interfere with MEF2 or MEF2-regulated transactivators. Taken together, these data are consis-
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 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 transactivation 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 γ-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 homebox 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 C 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 Gbxysen 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.

We specially thank Yukari Yamashita, Tammi S. Seaman and Ma Jesús Andrés for expert technical assistance. We are indebted to Paul E. Neumann for invaluable and generous help, to Karen Blochlinger for discussions and gift of the F2 antisera, to Jane E. Aubin for the RCJ 3.1C5 cells, and to Bjorn R. Olsen for helpful suggestions and discussions. We also thank Ruth Steinbrich for excellent assistance in the preparation of antigens and initial staining. We are grateful to Jay W. Schneider, Roger E. Breitbart, Hanh Nguyen, and Steve A. Mayer for critical reading of the manuscript. This work was supported in part from grants of the NIH and HHMI. V.A. is the recipient of a Postdoctoral Fellowship from the Spanish MEC.

**References**


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


(Accepted 12 July 1992)

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.