Molecular Vision 3 : 7, 1997 <http://www.emory.edu/molvis/v3/dhawan>
Received 9 Apr 1997 | Accepted 6 June 1997 | Published 10 June 1997

Isolation and Expression of Homeobox Genes from the Embryonic Chicken Eye

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Purpose: To identify homeobox-containing genes that may play a role in the differentiation of ocular tissues.
Methods: Total RNA was isolated from microdissected chicken embryo eye tissues at 3.5 days of development (embryonic day 3.5; E3.5). An "anchor-oligo-dT primer" was used for the synthesis of cDNA. Degenerate oligonucleotides designed from highly-conserved sequences in the third helix of the homeobox and the "anchor-primer" were used to amplify cDNAs by polymerase chain reaction (PCR). PCR products were cloned and sequenced. The spatial and temporal expression of selected transcripts was mapped by whole-mount in situ hybridization and northern blot analysis.
Results: After sequencing eighteen clones we identified a member of the distal-less family (dlx-3) in cDNA from presumptive neural retina and three chicken homologs of the Xenopus "anterior neural fold" (Xanf-1) in cDNA from anterior eye tissue. Dlx transcripts were mapped by in situ hybridization. Expression began at Hamburger and Hamilton stage 14 (E2.5) and was widely distributed in embryonic mesenchyme on E3 and E4. Expression increased in the retina during early development and persisted until after hatching. The one anf clone selected for further study was not detected by in situ or northern blot analysis.
Conclusions: It is feasible to isolate homeobox cDNAs directly from microdissected embryonic tissues. Chicken dlx-3 mRNA has a wider distribution in the embryo than expected, based on the expression of the mouse homolog. Dlx-3 may play a role in establishing or maintaining the differentiation of the retina.

Homeobox genes control pattern formation and position-specific cell differentiation in developing embryos (1-5). The proteins encoded by the homeobox superfamily contain a highly conserved DNA-binding domain, the homeodomain, comprised of four alpha helical segments. The second and third helices form a helix-turn-helix motif involved directly in DNA binding. The first and the fourth helixes are believed to contribute to the specificity of DNA-binding (5, 6)

In the last decade, several families of homeobox genes have been identified. These genes were often identified using conserved sequences from the homeobox as probes to screen cDNA libraries. (1, 7, 8) Within a homeobox family, the amino acid sequence of the homeodomain is highly conserved . Outside the homeodomain there is usually sequence similarity between members of a family, but little similarity to other homeobox families.

Our laboratory has been interested in identifying the factors responsible for the regional specification of the optic vesicle during eye formation. We have focused our attention on the formation of the ciliary epithelium, a double-layered epithelium that differentiates near the margin of the optic cup, adjacent to the lens. Although hybridization experiments have identified homeobox genes selectively expressed in this region (9), homeobox-containing transcripts rarely are isolated directly from differentiating eye tissues. We used degenerate PCR primers from the third helix of the homeobox and an oligo-dT-anchor primer to amplify and isolate homeobox-containing sequences in cDNA prepared from ocular tissues microdissected from a small number of stage 22 (embryonic day 4; E4) chicken embryos (10). The utility of this approach is demonstrated by the identification of new homeobox sequences, one isolated from presumptive retina and the others from the anterior optic cup and surrounding mesenchyme. The expression of one of these transcripts was mapped by whole-mount in situ hybridization.

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MATERIALS AND METHODS

Chicken Embryos—Fertilized chicken eggs were obtained from Truslow Farms, Chestertown, MD. Embryos were staged according to Hamburger and Hamilton (10). The experiments reported were conducted according to the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, DHEW Pub. No. (NIH) 78-23 and the ARVO Guidelines for the Use of Animals in Vision Research.

Cloning Strategy— Four embryonic eyes at stage 22 (E4) were dissected as described previously (11) to obtain neural retina, anterior optic cup, and other eye tissues. The anterior optic cup included the presumptive ciliary epithelium and anterior periocular mesenchyme. Total RNA was extracted bythe method of Chomczynski and Sacchi (12). Approximately 5 µg of total RNA was obtained from the anterior optic cup and 1.7 µg from the neural retina. The T7-RNA polymerase promoter sequence (Table 1) tailed with 15 thymidine residues (dt15-T7; Fig. 1), was used as a primer to convert 1 µg of total RNA into cDNA with MMLV reverse transcriptase (Life Technologies, Gaithersburg,

Table I

A. Nucleotide sequences of oligos used in amplification of homeobox specific sequences from embryonic eye tissues. B. and C. Primer sets used to generate chdlx-3- and chanf-specific probe templates for in situ hybridizations.

A	Homeobox-specific PCR:		
1	#2606	HOX-A	5'GT(GC)AA(GA)AT(CT)TGGTT(CT)CA(GA)AA3'
2	#2607	HOX-B	5'AA(GA)AT(CT)TGGTT(CT)CA(GA)AA(CT)CG3'
3	#4661	T7-promoter sequence	5'AATACGACTCACTATAGGG3'
B	Distal-less-specific:		
	Set# 1		
1.	#3779	Sense	5'AGCCCCAACAACAGCGATT3'
2.	#5731	4534 + T7 promoter	5'AATACGACTCACTATAGGGTATTGACAGAGGTGTGGGC3'
	Set# 2		
1.	#4534	Antisense	5'TATTGACAGAGGTGTGGGC3'
2.	#4416	3779 + T3 promoter	5'AATTAACCCTCACTAAAGGGAGCCCCAACAACAGCGATT3'
C	Anterior neural fold-specific:		
	Set#3		
1.	#3780	Sense	5'TAATTCCACCTCCAGCCTG3'
2.	#5732	4659 + T7 promoter	5'AATACGACTCACTATAGGGCCTCTCCCAGCAAATTAAG3'
	Set#4		
1.	#4659	Antisense	5'CCTCTCCCAGCAAATTAAG3'
2.	#4657	3780 + T3 promoter	5'AATTAACCCTCACTAAAGGGTAATTCCACCTCCAGCCTG3'

MD). Degenerate primers (Hox A and Hox B, 32-fold degeneracy; Table 1) were derived from the sequence of the conserved third helix of the homeobox to serve as sense primers for PCR (Fig. 1). Amplification was first performed with the Hox A primer (Table 1; Fig. 1) as the sense and the T7 promoter sequence with the T-tail as the antisense primer. Approximately 80 ng of cDNA were used as template in a "hot-start" PCR with 2.0 mM magnesium chloride in the PCR buffer. Reactions were heated to 80˚C for 1 min 30 sec, Taq polymerase was added and the reaction was cycled 30 times at 94˚C, 1 min, 50˚C, 1 min and 72˚C, 1 min, with a final extension at 72˚C for 5 min. A second, nested PCR was performed with primer Hox B and the T7 promoter sequence without the T-tail using 2 µl of the previous PCR reaction as template at the following conditions: hot start, 80˚C, 1 min 30 sec, 96˚C 40 sec, 55˚C, 1 min, 72˚C, 1 min for 30 cycles, extension at 72˚C, 5 min . The PCR products from the second amplification were purified on

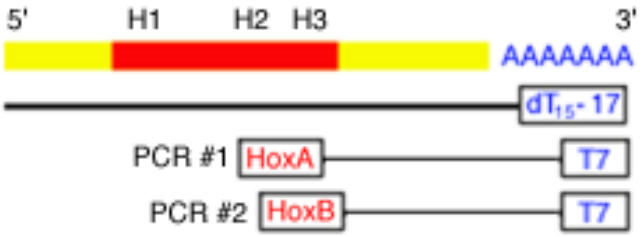

Figure 1. Schematic illustration of the strategy adopted to isolate homeobox genes from the eye tissues.Total RNA was extracted from micro-dissected eye components from four D3.5 eyes. Complementary DNA synthesis was primed with oligo-dT-tailed promoter sequence for the T7-RNA polymerase (dT15-T7) and homeobox specific cDNAs were amplified by PCR. Degenerate primers, Hox A & B, were synthesized from the highly conserved helix 3 (H3) region of the homeobox, to serve as sense primers for PCR. The Hox B primer overlapped most of the sequence of Hox A, as it was shifted in the 3-prime direction by only three bases. The first round of amplification (PCR#1) was done using primer Hox A and T7 promoter sequence and the second round (PCR#2) was carried out with primer Hox B and the T7 promoter sequence. The amplified PCR products were then cloned and sequenced.

GlassMax (Life Technologies) and directly cloned into Invitrogen's PCR-II T-A cloning vector (Invitrogen, San Diego, CA). Insert size was determined by restriction enzyme digestion and clones greater than 200 bp in length were sequenced in both directions by the ABI-fluorescent dye terminator method (Perkin-Elmer, Norwalk, CT). Sequence homologies were identified by searching Genbank.

A gel showing the PCR products obtained from several eye tissues from stage 22 embryos is shown in Figure 2. Each tissue produced a unique pattern of PCR products. In the present study individual bands were not excised from the gel and cloned. However, in future studies tissue-specific homeobox sequences could be obtained with this approach.

Synthesis of Probes—Sense and antisense single stranded DNA and RNA probes were generated from selected clones. A 130 bp PCR product from a clone related to the dlx-3 subfamily (chdlx-3) and a 222 bp product from a clone related to Xenopus Anf-1 (chanf) were amplified using specific primers (Fig. 3A & B; Table 1). These PCR products served as templates in single-stranded PCR to generate ^{32}P-labeled anti-sense probes (11) for screening northern blots. To prepare riboprobes for in situ hybridization, the PCR probe templates were re-amplified with modified primers that included the promoter sequence for T7 or T3 RNA polymerase (Table 1) to directly attach the promoter sequences to the probe templates. Digoxigenin-labeled sense or antisense riboprobe templates were produced in separate reactions using the Genius 4 RNA labeling kit (Boehringer-Manneheim, Indianapolis, IN). *Northern Blot Analysis*—Total RNA was extracted from the tissues of interest by the method of Chomczynski and Sacchi (12). RNA pellets were re-precipitated with 4M lithium chloride and 10 µg aliquots of total RNA were resolved on 1% formaldehyde gels. The blotted RNA was probed with the anti-sense strand of the probes labeled with 32P by single-stranded PCR (11).

Whole-Mount In situ Hybridization— Whole-mount in situ hybridization with digoxigenin-labeled probes was done by modified methods based on Harland (13) and Li et al (14).

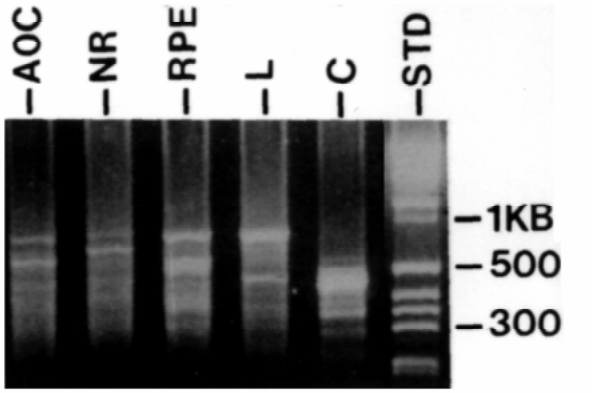

Figure 2. An agarose gel showing the PCR products generated from several ocular tissues. Tissues were isolated from stage 22 embryos, RNA was extracted, reverse transcribed and amplified with two sets of primers, as shown in Fig. 1. The pattern of bands obtained from each tissue was unique. AOC, anterior optic cup; NR, presumptive neural retina; RPE, retinal pigment epithelium; L, lens; C, corneal epithelium; STD, molecular weight standards.

Briefly, embryos were fixed overnight at 4˚C in MEMFA (0.1 M MOPS, pH 7.4; 2 mM EGTA; 1 mM magnesium sulfate and 3.7% formaldehyde), rinsed in 0.9% saline and stored in 90% methanol at -20˚C. For in situ hybridization, embryos were rehydrated, treated with proteinase K (10 µg/ml in PBS, 0.1% Tween-20) for 15 minutes at room temperature, acetylated and hybridized as described by Li, et al. (28). Hybridization was carried out overnight at 58˚C for chdlx-3 and 60˚C for chanf probes. Probe concentration was approximately 0.2 µg/ml. Embryos were washed at the same temperatures in 50% formamide, 2xSSC; 0.1% CHAPS and 50 mM glycine, pH 7.2 and treated with RNAase A and T1 at 37˚C for 1 hr. The last wash before blocking was done in maleate buffer pH 7.5. After blocking for two hours at 4˚C, embryos were incubated overnight at 4˚C with alkaline phosphatase-conjugated anti-digoxigenin antibody (1/2000; Boehringer-Manneheim), then washed extensively with BSA-saline. Color development used NBT and BCIP as substrates for alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) for chdlx-3 probes and either NBT/BCIP or BM-purple (Boehringer-Manneheim) for chanf. The NBT/BCIP substrate was prepared in Tris buffer (pH 9.5), containing 10% polyvinyl alcohol of molecular weight 13,000-23,000 (98% hydrolyzed; Aldrich, Milwaukee, WI) (15).

RESULTS

Sequence Comparisons— Of 5 clones isolated and sequenced from the neural retina, one containing a 228 bp insert showed homology to the third distal-less sub-family (Fig. 4A). We refer to this sequence as chicken dlx-3 (chdlx-3). As is evident from Fig. 4A, maximum amino acid sequence homology of chdlx-3 was to mouse dlx-3, zebrafish brdlx-3, and another member of the dlx-3 family, newt nvhbox4. Sequences from two other Urodeles, Pleurodeles waltii (Genbank accession #U49645) and Ambystoma mexicanum (Genbank accession #U59480) were identical to nvhbox3 and are not shown.

A

```
2607(Hox B)->                                                        48
AAG ATC TGG TTT CAA AAC CGC CGC TCC AAG TTC AAG AAG CTC TAC AAG
 K   I   W   F   Q   N   R   R   S   K   F   K   K   L   Y   K
                                3779->                               96
AAC GGC GAG GTG CCG TTG GAG CAC AGC CCC AAC AAC AGC GAT TCC ATG
 N   G   E   V   P   L   E   H   S   P   N   N   S   D   S   M
                                                                    144
GCC TGC AAC GAA GGA ACT GCT TTT GCG GGC GTT GAG GGA CTG CGT GTG
 A   C   N   E   G   T   A   F   A   G   V   E   G   L   R   V
                                                                    192
AGG GTT GCG AAG GGA CTG CTC CAC ACC TGC TGC TGT GCA GCC CAC ACC
 R   V   A   K   G   L   L   H   T   C   C   C   A   A   H   T
       <-4534                              223
TCT GTC AAT AAA CAC CTT TTG GTC TTT GTT TAA AAA AAA AAA AAA A-T7
 S   V   N   K   H   L   L   V   F   V   *
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B

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2607(Hox B)->                                                        48
AAG ATC TGG TTC CAG AAC CGT CGT GCA AAA CTG AAG AGA TCC CAC CGA
 K   I   W   F   Q   N   R   R   A   K   L   K   R   S   H   R
                                3780->                               96
GAA TCA CAG TTT TTA ATG GTG AAA AAT AAT TCC ACC TCC AGC CTG CTA
 E   S   Q   F   L   M   V   K   N   N   S   T   S   S   L   L
                                                                    144
GAG TAG GAG GAA GGA TGG CTT GCC TCG ATA TTA CTA CAA TGG GAC TTG
 E   *
                                                                    192
AAG AAT TAC TGG CAT TGG CTG TAA TGT GTA TTA AAC AAA TGA TTA TGT
                                                                    240
ATT AAT TAT GGA TTC CAT CCC TAG ATT TGA AAG ATT ATC AAT CCC CCG
                                                                    288
CTC AAA TTA ACT GTA AAT ATG TGA ACG TAT TGT TAA ACT TAA TTT GCT
  <-4659                                                            336
GGG AGA GGA AAG GTG GAA CTC TCT CCT TAT ATT TTT TTC GAT TTC ATA
                              360
TTT TGT CCA CGA ACC GGC ACA TTG AAA AAA AAA AAA AAA-T7
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Figure 3. The nucleotide and amino acid sequences of A) the chicken Distal-less (Chdlx-3) and B) the anterior neural fold (Chanf) cDNA fragments isolated from extracted ocular RNA . The nucleotide and amino acid sequences of A) the chicken Distal-less (Chdlx-3; GenBank accession # U31667) and B) the anterior neural fold (Chanf; GenBank accession # U31800) cDNA fragments isolated from RNA extracted from the presumptive neural retina and the anterior optic cup of stage 22 embryos. Sequences in bold are within the homeobox. Positions of primer sequences used in the study are underlined and the arrows indicate the direction of the primers. Primers 2607 (Hox B) and T7 were used to amplify the homeobox sequences. For chdlx-3, primers 3779 and 4534 (A) and for chanf, primers 3780 and 4659 (B) were used to generate the templates for the probes used for in situ hybridizations and Northern blots.

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A
        Homeodomain----(        Carboxyl terminus

        helix-3       helix-4          6
mdlx3   KIWFQNRR        SKFKKLYK        NGEVP.LEHSPNNSDSMACNSPPSPALWDTSSHSTPAPARNPLPPPLPYSASPNYLDDPTTSWYHTQNLSGPHLQQQPPQPATLHHASPGPPPNPGAVY|
zdlx3   ********        ********        *****.******A*************V**NNA**SQVNRGQIPQ***SSTPPYMEDYSNHWYQQGSHLQHPV*HPGP*QSVGAVY|
nvhbox4 ********        ********        *****GM***D*********A**T*****TP*RVQHTQAQPL*HNSSPSYLEDYNPWYHHPQNLSGHLQPPGTMHHTPPGTGAVY|
chdlx3  ********        ********        *****.**************EGTAF*GVEGLRVRVAKGLLHTCCCAAHT*VNKHL*VFV|
chdlx5  ******K*        **I**IM*        ***M*.P****SS**P****SPQSP*VW*PQGSSRSIGHHGHGH**AANPSPGS**ES*SAWYPAASP*GSH?QPHGSLOHPLALPSGTIY|
mdlx2   ********        *****MW*        S**I*.T*QH*GA*A*PP*A***VS*PASWDFGAPQRM*GGGPGSGGGGAG*SGSSPSSAA*AFLGNYPWYHQASGSASHLQATAPLLHPSQTPQAHHHHHHHHHHAGGGAPVSAGTIF|
mdlx1   ******K*        ******M*        Q*GAA.**G*ALANGRALSAGS*PVPPGWNPNSSSGKGSGSSASGSYV*SYT*WYPSAHQEAMQQPQLM|
dll     ********        **Y**MM*        AAQG*GTNSGMPLGGGGPNPGQHSPNQMHSGGNNGGGSNSGSPSHY**PGH**TPSST*VSELSPQFPPT*LSPPT*A*WDQKP*WIAHK***QM*GYVPQYWYLPETNPSLVTVWPAV|


B
        Homeodomain----(        Carboxyl-terminus

        helix-3       helix-4
xanf-1  QIWFQNRR        AKLKRSHR        ESQFLIVKDSLSSKIQE|
xanf-2  ********        ********        **************E*|
mRpx    ********        **M***R*        *****MA*KPFNPDLLK|
Hesx-1  ********        **M***R*        *****MARKPFNPDLLK|
chanf   ********        ********        *****M**NNST*SLLE|
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Figure 4. Amino acid sequences of chdlx-3 and chanf mRNA fragments aligned to other dlx- and anf sequences to show homologies. Sequences identical to mdlx-3 are shown in asterisks (*) and stop codons as vertical hashes (|). The GAVY sequence at the end of other dlx-3 sequences is shown in blue. Chdlx-3 and chanf are denoted by agenta text. **A.** The zebrafish (zdlx-3), the newt (nvhbox4), the chicken (chdlx-3 & 5), the mouse sequences (mdlx-1 & 2), and the Drosophila (dll), are shown aligned to the mouse mdlx-3 sequence. The single amino acid insertion of glycine in the Drosophila and newt sequences at the sixth position after the homeodomain is represented as a dot in the other sequences. The chdlx-3 sequence shows 24% homology to the mouse mdlx-1, 27% to mdlx-5, 31% to mdlx-2, 36% to mdlx-3, 33% to zdlx-3 and 29% to nvhbox4 in the sequences shown. **B**. The chicken anf (chanf) sequence aligned to the Xenopus (xanf-1 & 2) and mouse (Hesx-1, Rpx) sequences. The chicken sequence is identical to the xanf-1 & 2 in the homeobox sequences and is identical at 55% of the amino acid positions in the 3' coding sequences. It bears 87% identity to the mouse sequences in the homeobox region and 47-52% identity in the 3' coding sequences.

Sequences within the homeodomains of these genes were identical at the amino acid level. In the sequences flanking the homeodomain, chdlx-3 matched mdlx-3 in 21 amino acids and zdlx-3 in 19 amino acids. No significant homology was seen in the remainder of the coding sequences (Fig. 4A). Alignment with mouse dlx-1 and 2 and the Drosophila homolog, dll, revealed that chdlx-3 shared little amino acid sequence similarity beyond the homeodomain.

From the presumptive ciliary epithelium and periocular mesenchyme 13 clones were selected for sequencing. One, AOC19, had a 355 bp insert that was identical in protein sequence to Xenopus Anf1 in the homeodomain and 50% amino acid identity in the short, 18-amino-acid 3' coding region. The comparatively long 3' untranslated region contained seven in-frame stop codons. We refer to this clone as chicken anf (chanf). We identified two other clones among the 13 sequenced that shared 60-69% amino acid identity with chanf (data not shown). The remaining ten clones did not contain sequences related to homeobox genes.

Northern Blots— Northern blots probed with a ^{32}P-labeled chdlx-3 fragment identified a single band at approximately 1.5 kb (Fig. 5A & B). The mRNA for this gene was expressed in the heads of chicken embryos at stage 17 (E3; Fig. 5A). At stage 22 (E4), dlx-3 transcripts were abundant in the head but were barely detectable in the neural retina, anterior optic cup and limb bud (data not shown). Chdlx-3 transcripts were readily detectable in the retinas of E8 and E18 embryos and 2-day post hatch (P2) chicks (Fig. 5B) and were present at low levels in corneas and scleras of E8 and E18 embryos (data not shown). Stripping and reprobing the blots for chicken beta-actin (lower bands Fig. 5A & B) showed that similar amounts of RNA were loaded.

No chanf message was detected by northern blot analysis of total RNA extracted from whole heads of embryos at stages 17-22 (E3-4). Chanf sequences were not detected in RNA isolated from microdissected retina, anterior optic cup, retinal pigmented epithelium or pituitary primordium from stage 22 (E4) embryos (data not shown). revealed that the most intense staining in the head was in the mesenchyme beneath the floor of the hindbrain (Fig. 6I), the ectodermal lining of the otic vesicle (Fig. 6I), the mesenchyme of the mandibular arch (Figs. 6I and 6L) and the cranial ganglia (Fig. 6I). In the leg buds, chdlx-3 transcripts were expressed in the avascular mesoderm and in the dorsal ectoderm (Figs. 6K and 6M). Transcripts were also detected in the mesenchyme surrounding the neural tube, in the scelerotome and in the mesonephros, as shown in Fig. 6K.

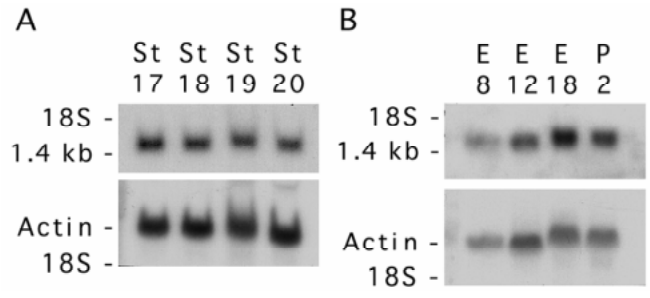

Figure 5. Northern blot analysis of the chicken dlx-3 and beta-actin transcripts. A. Blots of total RNAs extracted from the heads of embryos at stages 17, 18, 19, and 22 (embryonic days 3-4) of development. B. Blots of the total RNAs extracted from retinas at embryonic day 8 (E8), E12, E18, and 2 days post hatch (P2). Ten µg of RNA was resolved on 1% formaldehyde gel. The chdlx-3 transcripts were approximately 1.5 kb (top) and the chicken beta-actin approximately 2.4kb (bottom).

As with northern blot analysis, whole mount in situ hybridizations for chanf transcripts in embryos from stage 10 through E6 did not reliably show detectable levels of this message. It may be necessary to isolate longer cDNA sequences or to employ more sensitive detection methods to evaluate the expression pattern of this mRNA.

DISCUSSION

Direct Amplification and Cloning of Homeobox cDNA Sequences— Homeobox genes control pattern formation and position-specific cell differentiation during development. Because they are known to have expression patterns localized to sharply-delimited domains, we decided to clone homeobox genes directly from differentiating eye tissues. Our aim was to isolate messages that could be used as markers in experiments designed to identify the signals that orchestrate the regional differentiation of the eye.

Whole Mount In Situ Hybridization— Chdlx-3 transcripts were first detected at low levels in the optic cup at stage 14 (Fig. 6A). At stage 18 (E3, Fig. 6C) expression was seen in the optic cup, forebrain, branchial arches, and the limb buds. About one day later, at stage 22, chdlx-3 transcripts were more abundant in the optic cup (Figs. 6E, 6H) and the branchial arches (Fig. 6E). At this stage intense staining was also seen in a group of cells in the region between the nasal placode and the optic cup (Fig. 6G). A lower level of staining was seen in the mesenchyme surrounding the frontal lobes of the brain at all of these stages. Vibratome sections of stage 22 embryos In recent years, amplification of complementary DNA by RT-PCR has made it easier to isolate cDNA sequences from small amounts of embryonic tissues. Researchers have employed this technique to amplify cDNA fragments between two highly conserved domains in a gene family, then used the fragments generated as probes to isolate the rest of the sequence by screening cDNA libraries (16) or using rapid amplification of cDNA ends (RACE) (17, 18) Although this approach has resulted in the identification of a large number of genes belonging to families such as the kinases (19-20), phosphatases (21, 22), and homeoboxes (16, 18, 23), it has its limits. Most often, the amplified cDNA fragments contain sequences that are quite similar to each other, thereby limiting their use as probes for studying patterns of differential gene expression. Because mapping distribution patterns by in situ hybridization was critical for our experiments, we opted to amplify sequences between a highly conserved region in the homeobox and the 3'-end of the messenger RNAs. This would provide sufficient sequence information to identify homologs in other species, while providing sequences from the 3'-untranslated region for use as specific probes.

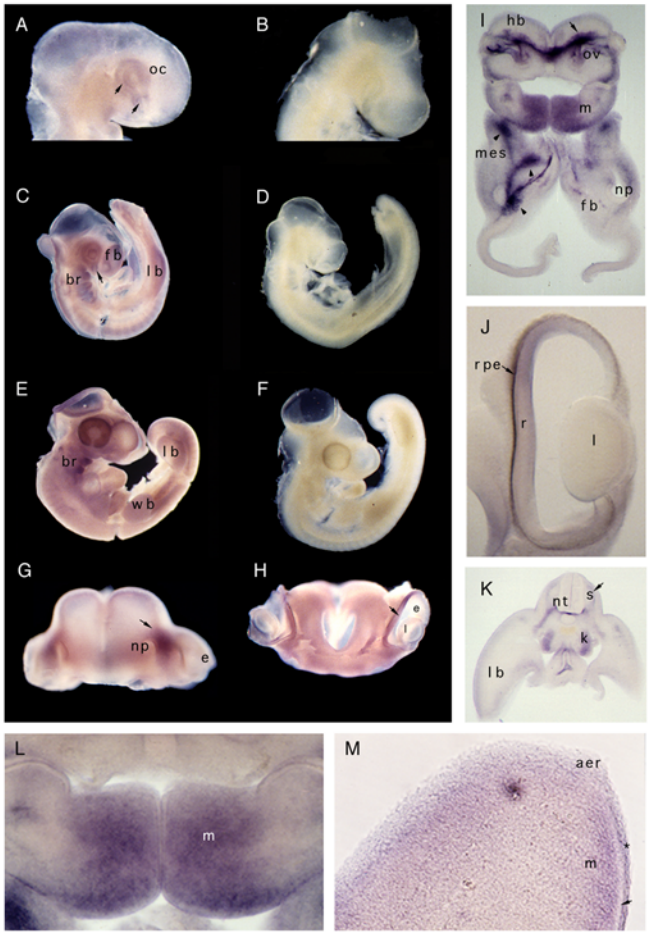

Figure 6. Whole mount in situ hybridization of chicken embryos, probed with digoxigenin labeled chdlx-3 riboprobes. A, C, E, G, and H were probed with the anti-sense strand and B, D, and F were probed with the sense strand. The embryo in A shows dlx- transcripts in the optic cup (arrows) at early D3 (stage 16) of development. In C, at stage 18 of development, the chdlx-3 transcripts were present in the forebrain (fb), the optic cup (arrow), the branchial arches (br), and the leg bud (lb). At 3.5 days (stage 22, embryo in E), chdlx-3 transcripts were more abundant in the optic cup and the branchial arches. Staining is also seen in the limb buds (lb and wb). G is the anterior view of the frontal lobes and the nasal placode of a D3.5 embryo. Intense staining is seen in the mesenchyme in the region between (arrow) the eye (e) and nasal placode (np). Faint staining is also seen in the whole frontal lobe. H is the posterior view of the tissue in G, showing low levels of staining in the mesenchyme of the frontal lobes and more intense staining in the retina (arrow). I, J and K are 70 µm Vibratome sections of stage 22 embryo. I is through the floor of the hindbrain (hb), otic vesicle (ov), mandibular arch (m), the nasal placode (np), and the forebrain (fb). Intense staining is seen in the mesenchyme beneath the floor of the hindbrain (arrow), in the mandibular arch (m), and the cranial ganglia (arrow heads). J is a section through the right eye in H, showing staining in the retina. K is a transverse section through the trunk at the level of leg buds. Staining is seen in the migrating neural crest (arrow), in the mesonephros (k) and in the peripheral avascular zone of the mesoderm in the leg bud. L and M are higher magnification (20X) views of similar sections as in I and K. L shows that staining in the mandibular arch is restricted to the cells of the mesenchyme. In M localization of chdlx-3 transcripts in the dorsal ectoderm (*) and the peripheral avascular mesenchyme (m) of the limb is apparent. The unstained region under the dorsal ectoderm is clearly distinguishable (arrow). The apical ectodermal ridge (aer) was not stained appreciably.

The sequences in the third helix of the homeobox were most suited to our purpose, as these are the most highly conserved among homeobox families. PCR products generated from this region also include a portion of the 3' end of the homeobox sequence, permitting positive identification of homeobox-containing clones. We primed the cDNA reactions with 15 thymidine residues anchored to the T7 promoter sequence to ensure maximal representation of the messages present in the tissues of interest and amplified the cDNAs with degenerate, overlapping primers (Hox A and Hox B) to increase the probability of obtaining homeobox-specific products.

Our results show that this method was capable of identifying new homeobox sequences. The chdlx-3 and the chanf sequences are identical to their homologs from other species in the homeobox region and have a high degree of homology in their 3'-coding sequences.

Although both sequences include much or all of the 3'-coding region, the chdlx-3 clone comes to an abrupt end at what appears to be a stop codon. Because this stop codon is TAA, it is possible that one or both of the adenines were contributed by the poly-A primer. If this occurred, some of the 3'-coding sequence may be absent. This explanation may be correct, because the coding sequence we obtained for chdlx-3 is shorter than dlx-3 sequences from other species and because all other dlx-3 orthologs, but not chdlx-3, end in the amino acid sequence "GAVY." Although the chanf cDNA includes a substantial 3' untranslated region, it also may be truncated at its 3' end, because no canonical poly-A addition signal is present near the 3' end of the clone.

Absence of the 3' end of a cDNA, as discussed above, may be caused by priming the cDNA from short stretches of adenines. Errors of this type may be avoided in future studies by using shorter chains of thymines (6-7 Ts) for cDNA synthesis. The first round of PCR would then use an anchor primer linked to longer stretches of poly-T (18-22 Ts) at higher annealing temperatures for amplification. This would assure that only cDNAs initiated in the poly-A tail would be amplified.

In future studies we also suggest that one not use RNA polymerase promoter sequences as anchor-primers. Most cloning vectors already have these sequences and two identical sites in the plasmid created interference in screening and riboprobe synthesis. The use of newer thermostable polymerases and polymerase mixtures more suitable for amplification of longer PCR fragments might provide an added advantage in amplifying more of the 3' end of homeobox-containing cDNAs.

The Chicken Dlx-3 Sequence and the Distal-less Family—In Drosophila a single distal-less gene (dll) is expressed in early larval stages, where it controls the formation of the larval sense organs. After shutting off briefly in the later larval stages, dll is expressed again in the pupae during limb formation (24, 25).

Multiple distal-less homologs are found in vertebrate species. It has been suggested that the complex developmental

patterns in vertebrates are partially accounted for by the increased number and diversity of homeobox genes, compared to the complexity of this gene superfamily in invertebrates. This has apparently led to the expression of several members of a given vertebrate homeobox family in overlapping or complementary domains. Thus, in mice (16, 26-31), frogs (32-35), and zebrafish (36-38), multiple genes from the distal-less family are expressed in several regions of the brain, the olfactory, otic, and optic sense organs, the mesenchyme of the branchial arches, and the developing limbs. In newts, two distal-less genes are co-expressed in regenerating limbs (39). The single distal-less homolog identified in rat, rdlx (40), and its mouse ortholog, mdlx-5 (31), have wide expression domains in the forebrain, branchial arches, otic vesicles, and cranial ganglia, which they share with mdlx-1, 2 and 3 (16, 26-31, 40). The recently isolated chicken ortholog, chdlx-5 (41), is expressed in the AER in the limb, as are the members of the dlx-1 and 3 families. The dlx-5 genes in chicken, mouse, and rat also have a novel expression in differentiating cartilage. Based on these complex and overlapping expression patterns, it has been proposed that the vertebrate dlx family is important in craniofacial and sense organ development and in specifying proximo-distal position in limbs.

From Fig. 4A it is apparent that the partial chdlx-3 sequence bears maximum homology to the members of the dlx-3 sub-family. Based on the known expression pattern of members of this family in other vertebrates, we expected chdlx-3 transcripts to be expressed in the neural crest-derived mesenchyme of the branchial arches, in the apical ectodermal ridge (AER) at the distal tip of the limb, and in the olfactory and otic epithelia (30, 34, 38). Although transcripts were present in many of these areas, the chicken dlx-3 homolog has a wider expression domain than its counterparts in the mouse, zebrafish, or frog. Whole-mount in situ hybridization revealed chdlx-3 expression in the forebrain, cranial ganglia, and mesonephric clusters. This pattern more closely resembles the distribution of members of the dlx-2 sub-family (16, 29, 30). Similarly, expression of chdlx-3 in differentiating neural retina is more like the expression of members of the dlx-1 sub-family (27, 37). Unlike other members of dlx-3 sub-family, chdlx-3 is expressed in the dorsal ectoderm and the mesoderm of the developing limbs where it appears to partially overlap the expression domain of chicken dlx-5 (41). Chdlx-3 was not detected in the AER, although, in the mouse dlx-1, 3, and 5 are all found in this region (27, 30). Given the unexpectedly wide and divergent expression of chdlx-3, it will be of interest to compare the expression patterns of the other chicken dlx family members with the distribution of their mouse, frog, and zebrafish orthologs.

Distal-less function— Drosophila distal-less specifies patterns along the proximo-distal axis of sensory appendages such as the maxilla, antenna, labrum, labia, and the thoracic limbs (23-25, 42). In this species, distal-less functions in a cell-autonomous manner and absence of distal-less causes appendages to grow without differentiation (25).

Information about the function of the distal-less family in vertebrates was provided by creating mice lacking dlx-2 by homologous recombination (51). In these animals, the development of the forebrain was abnormal and the morphogenesis of skeletal elements derived from the proximal regions of the first and second visceral arches was disturbed. The expression patterns of several homeobox genes, normally co-expressed with dlx-2, was not disturbed in dlx-2 knockouts. This suggested that dlx-2 was not responsible for the regional specification of the forebrain. However, in contrast to normal mice, the enzyme tyrosine hydroxylase was absent from neurons in the olfactory bulb in eight independent lines of dlx-2 null homozygotes. Dlx-1 continues to be expressed in these cells. This suggests that dlx-2 regulates the state of differentiation or the identity of these cells. If these characteristics of dlx-2 are typical of other members of the distal-less family, these genes may be more important in determining neuronal cell type or cell-specific characteristics than in defining regional identity. Dlx-2 null mice also had abnormalities in the differentiation of the proximal derivatives of the first and second branchial arch and in the membranous bones of the skull. It is not clear whether these abnormalities were due to defects in spatial patterning or cell differentiation. Derivatives of the distal portions of these arches were normal. This may be because dlx-3 and -5 were expressed in the distal regions of these arches (51).

The mouse genes for dlx-1 and dlx-2 map near the mutations Ul (ulnaless), which is characterized by abnormalities of the ulna, radius, tibia and fibula (43) and Far (First Branchial Arch), which is characterized by abnormalities in the first branchial arch and mandible (44), suggesting dlx-1 and dlx-2 as candidate genes for these mutations.

Although it is likely that members of distal-less family are responsible for cell differentiation in many complex tissue systems, such as the brain, branchial arches, otic vesicles, and the limbs, there is not yet enough information available about the functions of all the distal-less family members in the development of any vertebrate organ system to test this prediction. From the present study, the early and persistent expression of chdlx-3 in the retina suggests a function for this gene in retinal development.

A Chicken Homolog of the Anterior Neural Fold Gene— The anterior neural fold homolog identified in our studies is a member of a family of homeobox genes first reported in Xenopus (Xanf-1) (45). The mRNA for Xanf-1 is expressed in the neural ectoderm and is localized to the anterior region of the neural folds by mid-neurula. It is also expressed in the mesodermal cells comprising Spemann's organizer, a region responsible for determining the anterior-posterior axis in the frog embryo. Ectopic expression of Xanf-1 in early embryos revealed that it is able to control the positional identities of cells along the anterior-posterior axis and the development of the anterior mesodermal and ectodermal structures (45, 46). Homologs have now been isolated from a gastrula-specific Xenopus cDNA library (Xanf-2) (47) and in mice (Hesx-1 and Rpx) (48-50) and zebrafish. Mathers and coworkers reported that Xanf-2 is expressed in the early upper sensorial ectoderm (which gives rise to the stomodeum, the anlage of the pituitary gland), the optic vesicles, and the olfactory placodes (47). Hesx-1 isolated from cultured, pluripotent mouse embryonic stem cells and by RNase protection assay

was found to be most abundant in embryonic liver cells (48, 49). In situ hybridization demonstrated that Rpx, a closely related gene, was expressed in the early cephalic neural plate (presumptive prosencephalon region) and the endoderm underlying it. A day later, Rpx localized to the prosencephalon, and later to the oral ectoderm (pituitary anlage) (50).

The chicken homolog of anf was not reliably detected by northern blot or in situ hybridization in embryos from stage 10-25. In particular, we were unable to detect transcripts in the anterior optic cup tissues at E3.5, the tissue and stage from which the initial cDNA was isolated. Use of a longer clone and more sensitive detection methods might permit the localization of chanf transcripts more effectively. Our preliminary data and the results of others suggest that the Anf/Rpx family has several members in vertebrates. The role of these genes in eye formation and morphogenesis is not clear at present, although Xanf-1 and -2 are expressed in cells that will form the optic cup (47) and mice in which Rpx function is deleted by homologous recombination are microphthalmic or anophthalmic (KA Mahon, personal communication).

ACKNOWLEDGEMENTS

The authors thank Dr. Olof Sundin for his guidance with the whole mount in situ hybridization technique and Mr. Wayne Randolph and Ms Ida Lirette for their help with photography. Support for this work was from NIH grant EY04853 to DCB.

REFERENCES

1. Gehring, W. J. (1987) Homeo boxes in the study of development. Science 236:1245-1252.
2. Krumlauf, R. (1994) Hox genes in vertebrate development. Cell 78:191-201.
3. Lawrence, P. A., and Morata, G. (1994) Homeobox genes: Their function in Drosophila segmentation and pattern formation. Cell 78:181-189.
4. Kenyon, C. (1994) If birds can fly, why can't we? Homeotic genes and evolution. Cell 78:175-180.
5. Gehring, W. J., Affolter, M., and Burglin, T. (1994) Homeodomain proteins. Ann. Rev. Biochem. 63:487-526.
6. Scott, M. P., Tamkun, J. W., and Hartzel III, G. W. (1989) The structure and function of homeodomain. Biochim. Biophys. Acta 989:25-48.
7. Scott, M. P., and Weiner, A. J. (1984) Structural relationships among genes that control development: Sequence homology between the antennapedia, ultrabithorax, and fushi tarazu loci of Drosophila. Proc. Natl. Acad. Sci. USA. 81:4115-4119.
8. McGinnis, W., Garber, R. L., Wirz, J., Kuroiwa, A., and Gehring, W. J. (1984) A homologous protein-coding sequence in Drosophila homeotic genes and its conservation in other metazoans. Cell 37:403-408.
9. Beebe, D. C. (1994) Homeobox genes and vertebrate eye development. Invest. Ophthalmol. Vis. Sci. 35:2897-2900.
10. Hamburger, V., and Hamilton, H. L. (1951) A series of normal stages in the development of the chick embryo. J. Morph. 88:49-92.
11. Dhawan, R. R., and Beebe, D. C. (1994) Differential localization of collagen type IX isoform messenger RNAs during early ocular development. Invest. Ophthalmol. Vis. Sci. 35:470-478.
12. Chomczyski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.
13. Harland, R. M. (1991) In situ hybridization: An improved whole-mount method for Xenopus embryos. Methods in Cell Biol. 36:685-695.
14. Li, H., Yang, J., Jacobson, R. D., Pasco, D., and Sundin, O. (1994) Pax-6 is first expressed in a region of ectoderm anterior to the early neural plate: Implications for stepwise determination of the lens. Dev. Biol. 162:181-194.
15. Barth, J., and Ivarie, R. (1994) Polyvinyl alcohol enhances detection of low abundance transcripts in early stage quail embryos in a nonradioactive whole mount in situ hybridization technique. Biotechniques 17:324-328.
16. Robinson, G. W., Wray, S., and Mahon, K. A. (1991) Spatially restricted expression of a member of a new family of murine distal-less homeobox genes in the developing forebrain. The New Biol. 3:1183-1194.
17. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA. 85:8998-9002.
18. Weiss, K. M., Bollekens, J., Ruddle, F. H., and Takashita, K. (1994) Distal-less and other homeobox genes in the development of the dentition. J. Exp. Zool. 270:273-284.
19. Wilkie, T. M., and Simon, M. I. (1991) Cloning multigene families with degenerate PCR primers. Methods: A Companion to Methods in Enzymology 2:32-41.
20. Potts, J. D., Harocopos, G. J., and Beebe, D. C. (1993) Identification of receptor kinases in the embryonic chicken lens. Curr. Eye Res. 12:759-763.
21. Nishi, M. Ohagi, S., and Steiner, D. F. (1990) Novel putative protein tyrosine phosphatases identified by the polymerase chain reaction. FEBS Letters 271:178-180.
22. Chen, M. X., Chen, Y. H., and Cohen, P. T. W. (1992) Polymerase chain reactions using Sacharomyces, Drosophila and human DNA predict a large family of protein serine/threonine phosphatases. FEBS Letters 306:54-58.
23. Panganiban, G., Nagy, L., and Carrol, S. B. (1994) The role of the distal-less gene in the development and evolution of insect limbs. Current Biology 4:671-675.
24. Cohen, S. M., Bronner, G., Kuttner, F., Jurgens, G., and Jackle, H. (1989) Distal-less encodes a homeodomain protein required for limb development in Drosophila. Nature 338:432-434.
25. Cohen, S. M., and Jurgens, G. (1989) Proximal-distal pattern formation in drosophila: Cell autonomous requirement for distal-less gene activity in limb development. EMBO J. 8:2045-2055.
26. Price, M., Lemaistre, M., Pischetola, M., Di Lauro, R., and Duboule, D. (1991) A mouse gene related to distal-less shows a restricted expression in the developing forebrain. Nature 351:748-751.

27. Dolle, P., Price, M., and Duboule, D. (1992) Expression of the murine Dlx-1 homeobox gene during facial, ocular and limb development. Differentiation 49:93-99.
28. Porteus, M. H., Bulfone, A., Ciaranello, R. D., and Rubenstein, J. L. R. (1991) Isolation and chracterization of a novel cDNA clone encoding a homeodomain that is developmentally regulated in the ventral forebrain. Neuron 7:221-229.
29. Bulfone, A., Kim, H. J., Puelles, L., Porteus, M. H., Grippo, J. F., and Rubenstein, L. R. (1993) The Mouse Dlx-2 (Tes-1) gene is expressed in spatially restricted domains of the forebrain, face and limbs in midgestation mouse embryos. Mech. Dev. 40:129-140.
30. Robinson, G. W., and Mahon, K. A. (1994) Differential and overlapping expression domains of Dlx-2 and Dlx-3 suggest distinct roles for distal-less homeobox genes in craniofacial development. Mech. Dev. 48:199-215.
31. Simeone, A., Acampora, D., Pannese, M., D'Esposito, M. Stornaiuolo, A., Gulisano, M., Mallamaci, A., Kastury, K. Druck, T., Huebner, K., and Boncinelli, E. (1994) Cloning and characterization of two members of the Dlx gene family. Proc. Natl. Acad. Sci. USA. 91:2250-2254.
32. Asano, M., Emori, Y., Saigos, K., and Shiokawa, K. (1992) Isolation and characterization of a xenopus cDNA which encodes a homeodomain highly homologous to Drosophila distal-less. J. Biol. Chem. 267:5044-5047.
33. Dirksen, M. L., Mathers, P., and Jamrich, M. (1993) Expression of a Xenopus distal-less homeobox gene involved in forebrain and cranio-facial development. Mech. Dev. 41:121-128.
34. Morasso, M. I., Jamrich, M., and Sargent, T. D. (1993) The homeodomain gene Xenopus distal-less-like-2 (Xdll-2) is regulated by a conserved mechanism in amphibian and mammalian epidermis. Dev. Biol. 162:267-276.
35. Papalopulu, N., and Kintner, C. (1993) Xenopus distal-less related homeobox genes are expressed in the developing forebrain and are induced by planar signals. Development 117:961-975.
36. Ekker, M., Akimenko, M. A., Bremiller, R., and Westerfield, M. (1992) Regional expresion of three homeobox transcripts in the inner ear of zebrafish embryos. Neuron 9:27-35.
37. Westerfield, M., Akimenko, M. A., Ekker, M., and Puschel, A. (1993) Eyes, ears, and homeobox genes in zebrafish embryos. In: "Molecular Basis of Morphogenesis" New York: Wiley-Liss, pp 69-77.
38. Akimenko, M. A., Ekker, M., Wegner, J., Lin, W., and Westerfield, M. (1994) Combinatorial expression of three zebrafish genes related to distal-less: Part of a homeobox gene code for the head. J. Neurosci. 14:3475-3486.
39. Beauchemin, M., and Savard, P. (1992) Two distal-less related homeobox-containing genes expressed in regeneration blastemas of the newt. Dev. Biol. 154:55-65.
40. Zhao, G. Q., Zhao, S, . Zhou, X., Eberspaecher, H., Solursh, M., and Crombrugghe, B. D. (1994) rDlx, a novel distal-less-like homeoprotein is expressed in developing cartilage and discrete neuronal tissues. Dev. Biol. 164:37-51.
41. Ferrari, D., Sumoy, L., Gannon, J., Sun, H., Brown, A. M. C., Upholt, W. B., and Kosher, R. A. (1995) The expression pattern of the distal-less homeobox-containing gene Dlx-5 in the developing chick limb bud suggests its involvement in apical ectodermal ridge activity, pattern formation, and cartilage differentiation. Mec. Dev. 52:257-264.
42. Vachon, G., Cohen, B., Pfeifle, C., McGuffin, E., Botas, J., and Cohen, S. M. (1992) Homeotic genes of the bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene distal-less. Cell 71:437-450.
43. Davisson, M. T., and Cattanach B. M. (1990) The mouse mutation ulnaless on chromosome 2. J. Heredity 81:151-153.
44. Juriloff, D. M., and Harris, M. J. (1991) Mapping the mouse cranofacial mutation first arch (FAR) to chromosome 2. J. Heredity 82:402-405.
45. Zaraisky, A. G., Lukyanov, S. A., Vasilev, O. L., Smirnov, Y. V. Belyavsky, A. V., and Kazanskaya, O. V. (1992) A novel