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Differential Regulation of the Aldehyde Dehydrogenase 1 Gene in Embryonic Chick Retina and Liver*

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Aldehyde dehydrogenase (ALDH1) is highly expressed in the dorsal cells of the undifferentiated retina, where it has been proposed to play a role in the formation of a retinoic acid gradient along the ventrodorsal axis. In contrast to the retina, ALDH1 levels increase with differentiation in the liver and remain elevated in the adult tissue. To understand the molecular basis for differential expression of ALDH1 during development, we characterized the *ALDH1* transcripts expressed in chick retina and liver. By sequencing, primer extension, and S1 nuclease analysis, we show that retina *ALDH1* mRNA has an additional 300 nucleotides of 5′-untranslated sequence resulting from the transcription of two 5′ noncoding exons. There is a 24–29-kilobase pair (kb) gap between exons 1 and 2 and a 290-base pair gap between exons 2 and 3. Exon 3, which contains the ALDH1 start codon, represents the first exon of the liver transcript. Using a reporter gene assay, we have identified tissue-specific regulatory elements that govern *ALDH1* expression in primary retina and liver cultures. Constructs with >1.6 kb of DNA flanking the 5′-end of exon 1 showed elevated activity in retinal cultures but only basal activity in liver cultures. In contrast, constructs with <1 kb of 5′-flanking DNA were active in both retina and liver cultures. Our results suggest that an important mechanism for the control of *ALDH1* transcriptional activity is through the presence of inhibitory elements located 0.7–1.6 kb upstream of the *ALDH1* gene. DNase I footprint analysis reveal four sites of protein-DNA interaction within this region, one of which is specific to the liver and corresponds to a NF-κB/Rel binding site.

Development is accompanied by a finely orchestrated series of regulatory events that underlie cell- and stage-specific gene expression. Since the same gene product may be required in different cell types during different stages of development, numerous regulatory mechanisms have evolved to ensure correct gene expression at the appropriate developmental stages. Alternative 5′ exons, alternative transcription start sites, alternative promoters, alternative splicing, and cell-specific expression of transcription factors are all well documented mechanisms that allow genes to be expressed in a cell-, tissue-, or stage-specific manner.

Cytosolic aldehyde dehydrogenase (ALDH1)[1] has been proposed to play a variety of roles in the cell, including metabolism of neurotransmitters, detoxification of alcohol, and conversion of retinaldehyde to retinoic acid (RA) (1, 2). Consistent with a role in the oxidation of a wide variety of endogenous and exogenous aldehydes, ALDH1 is detected in fetal liver and kidney and is highly expressed in adult liver, kidney, skeletal muscle, and pancreas (3–5). ALDH1 is also highly expressed at early stages of retinal development; however, in contrast to liver, retinal differentiation is accompanied by a dramatic decrease in *ALDH1* mRNA (3, 6). Interestingly, ALDH1 (protein and mRNA) is restricted to the dorsal half of the developing mouse and chick retina (6–8). The sharp demarcation between *ALDH1*-expressing cells in the dorsal retina and nonexpressing cells in the ventral retina suggests a role for ALDH1 in the establishment of boundaries within the retina.

A second ALDH, ALDH6 or RALDH3, also involved in the conversion of retinaldehyde to RA, has recently been shown to be expressed in the ventral retina (9–11). Together, ALDH1 and ALDH6 are believed to form a ventrodorsal RA gradient that regulates the expression of RA-responsive genes critical for retinal development (8, 10, 12). Zebrafish embryos exposed to the inhibitor of ALDH activity, citral, at early stages of eye development lack a ventral retina (13). Conversely, zebrafish embryos grown in the presence of RA develop into larvae with eyes that appear duplicated along the dorsoventral axis (14, 15). These data support a role for ALDHs in retinal compartmentalization and patterning and suggest that RA may serve as a position-specific tag involved in guiding retinal axons to their correct targets in the brain (12, 16, 17). Because ALDH1 is expressed at early stages of retinal development, prior to axon formation, asymmetry in RA levels may be required to mark the different axes of the retina and to form the basis for retinotectal organization, as previously suggested for some Eph receptors and their ephrin ligands (18, 19).

To understand how regulation of the *ALDH1* gene is controlled during development, we studied the molecular basis for differential expression of the *ALDH1* gene in chick retina and liver. Here, we report that the *ALDH1* transcript expressed in the retina contains two additional noncoding exons compared with the *ALDH1* transcript expressed in the liver. We demonstrate that the sequences upstream of *ALDH1* exon 1 are transcriptionally more active in cells derived from the retina compared with liver, and we identify a region that is strongly inhibitory in liver cells. Our results indicate that the *ALDH1* gene is differentially regulated in chick retina and liver through usage of different transcription start sites and regulatory elements.

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[1] The abbreviations used are: ALDH, aldehyde dehydrogenase; RA, retinoic acid; CAT, chloramphenicol acetyltransferase; kb, kilobase pair; bp, base pair(s); nt, nucleotide(s); RACE, rapid amplification of cDNA ends.

MATERIALS AND METHODS

Library Screening—The chicken liver cDNA library in λgt11 and the chicken genomic library in EMBL-3 SP6/T7 prepared from adult chicken liver were purchased from CLONTECH. The chick retina cDNA library in λZAPII was prepared as described (3) except that the cDNA was produced from chick retina poly(A)$^+$ RNA at day 7 of incubation (day 7). For screening retina and liver cDNA libraries, ~2 × 10^5 bacteriophage were hybridized with a 1.5-kb *ALDH1* cDNA obtained from a day 3.5 chick retina library (3). Positive clones were purified by additional rounds of screening. Inserts from positive plaques obtained from the chicken liver cDNA library were subcloned into pBluescript, while pBluescript phagemids containing retina *ALDH1* cDNAs were excised from λZAPII using a helper phage (Stratagene). The chicken genomic library was screened with two *ALDH1* cDNA fragments: (i) a 350-bp *Eco*RI/*Bgl*II DNA fragment, most of which was unique to the 5′-end of retina *ALDH1* cDNA, and (ii) a 500-bp *Eco*RI/*Hin*dIII fragment, containing 5′ sequences shared between retina and liver cDNAs. Genomic DNA that either hybridized to the cDNAs or extended upstream of the cDNAs was subcloned into pBluescript. Sequences were generated using either the dideoxynucleotide chain termination method with T7 DNA polymerase (Amersham Pharmacia Biotech) or an ABI 310 automated sequencer. Regulatory elements were analyzed using the IFTI (available on the World Wide Web at www.ifti.org) and GeneTool programs (BioTools Inc., Edmonton, Alberta, Canada).

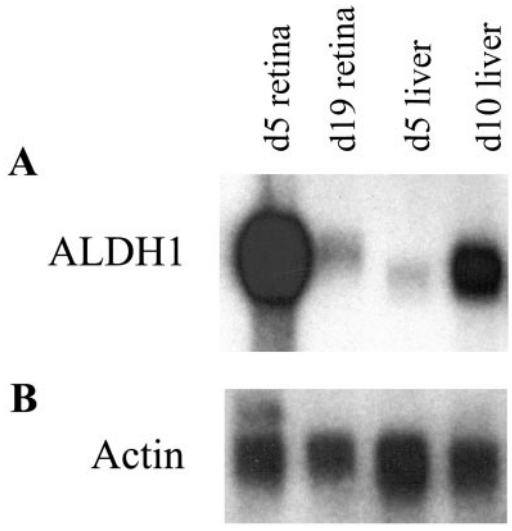

FIG. 1. **Northern blot analysis of *ALDH1* in chick liver and retina.** Two μg of poly(A)$^+$ RNA isolated from day 5 (*d5*) retina, day 19 (*d19*) retina, day 5 liver, and day 10 (*d10*) liver were electrophoresed in a 1.5% agarose-formaldehyde gel. The RNA was transferred to a nitrocellulose filter and sequentially hybridized with radioactively labeled chicken *ALDH1* cDNA (*A*) and actin cDNA (*B*).

Primer Extension—Poly(A)$^+$ RNAs were isolated from chick retina and liver as previously described (3). Primer 1 (5′-GAGATCCTTCAAGCAAACACA-3′), primer 2 (5′-CAGAGTACCAGTCATGACCTC-3′), and primer 3 (5′-GAGTGGTTCTGGCAGTGCTGGC-3′) (Fig. 2) were end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech) and T4 polynucleotide kinase. Each of the labeled primers was annealed to 2 μg of poly(A)$^+$ RNA at 45 °C for 90 min, and the cDNA was extended at 42 °C for 60 min using avian myeloblastosis virus reverse transcriptase (Promega). The primer extension products were heat-denatured and run on an 8% polyacrylamide gel containing 7 M urea in 1× TBE buffer. Marker lanes consisted of labeled sequencing reaction mixtures (G + A ladder) prepared according to Belikov and Wieslander (20) or end-labeled φX174 phage DNA digested with *Hin*fI.

S1 Nuclease Protection Assay—The S1 nuclease assay to map the 5′-end of the chick retina *ALDH1* transcript was performed as described by Favaloro *et al.* (21). The probe was prepared by digesting genomic DNA with *Sty*I, which cuts within exon 1 (Fig. 2). The ends were labeled with [γ-^{32}P]ATP (3000 Ci/mmol) and polynucleotide kinase, and the label from the upstream end was removed by cutting with *Bgl*II, generating a 478-bp fragment labeled (*) at the 3′-end. Two or four μg of poly(A)$^+$ RNA derived from liver or retina were hybridized with heat-denatured *Bgl*II-*Sty*I*-labeled probe in 80% formamide, 40 mM PIPES, 400 mM NaCl, 1 mM EDTA. Samples were incubated at 45 °C for 16 h and digested with 3000 units/ml S1 nuclease (Roche Molecular Biochemicals) for 60 min at 37 °C. The samples were precipitated with ethanol; resuspended in 80% formaldehyde, TBE buffer, 0.1% bromphe-

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                    ↓↓↓ ⇓    • *                              ∇
R                   CTCTTTTT TTTCCAAGTA TATCATCAAG ATCACACTCA ATTCAATGCC GATTATGTGT TTGCTTGAAG   68
                                                                          Primer 1

R  GATCTCTTTC TCTGAGTAAT ATTACTTGAT AAACACAGAA CTGAAGTAAG AATCTAAGCA TTCATTTGTT GCTGTTACTG AATCGTGCAT ATATAGAGGT  168

R  CATGACTGGT ACTCTGACAG TAGTGCTGAT CGCAGACCTT AGGGCAGAGT GCGCAGTGGA TTTGAAATAT TACAGACCTA GGAAATCAAG CCCTGATTGT  268
     Primer 2                                                                    Sty I

R  AAAGCTGCTT TCCAGTAAGT GAAGAAGTTC AATTAAAATC TGGGGACCTG CCTCCTGAGA GAAAGTAATT TTACAGGAGC GTACTCAAAC TCCTCACCTG  368
                           ↓                   ∇
L                          AGGAC AAAAATTGCT GCGCTTCCCT GATACCTCAT TTCACCTGTG TTT------- ---------- ----------   75

R  GCAACATGAA GAAGCAAGGC TCACCCAGCA ATCCTGCTCC TGTTTTGCCA GCACTGCCAG AACCACTCAA                              438
L  ---------- ---------- ---------- ---------- ---------- ---------- ----------                              145
                                                          Primer 3
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FIG. 2. **Sequence of the 5′-ends of liver and retina *ALDH1* cDNAs.** The 5′-ends of cDNA clones identified by screening chick retina and liver cDNA libraries are indicated by the *arrowheads*. Primer extension results are indicated by the *single arrows* (primers 1 and 3) and *double arrows* (primer 2). Primer sequences are shown in *boldface type*. The *closed circle* indicates the putative transcription start site of retina *ALDH1* mRNA as determined by S1 nuclease analysis using a DNA fragment labeled at the *Sty*I site (*double underlined*). The longest RACE product obtained extended to the nucleotide indicated by the *asterisk*. The start codon for both retina (*R*) and liver (*L*) ALDH1 is *underlined*. The GenBank™ accession number for the chicken *ALDH1* cDNA sequence is X58869.

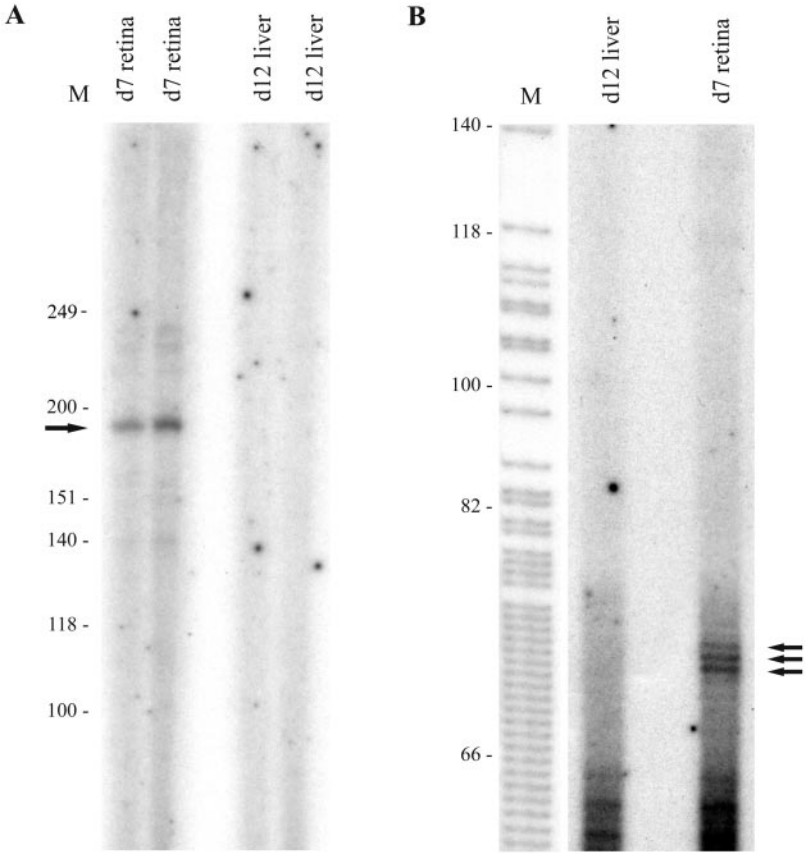

FIG. 3. **Identification of the 5′-ends of liver and retina *ALDH1* mRNAs by primer extension.** *A*, radioactively labeled primer 2 was annealed to poly(A)$^+$ RNA from day 7 (*d7*) retina (2 or 4 μg) and day 12 (*d12*) liver (2 or 4 μg) and extended using reverse transcriptase. *B*, radioactively labeled primer 1 was annealed to poly(A)$^+$ RNA from day 12 liver (2 μg) and from day 7 retina (2 μg). The products were run in an 8% denaturing polyacrylamide gel with end-labeled ϕX174 phage DNA digested with *Hin*fI (*A*) or a G + A sequencing ladder (*B*) as size markers. The *arrows* mark the positions of the primer extension products.

nol blue, and xylene cyanol; denatured at 90 °C for 2 min; and electrophoresed in a 7 M urea, 8% polyacrylamide gel in TBE buffer.

Rapid Amplification of cDNA Ends (RACE)—We used the AmpliFINDER RACE kit (CLONTECH) to extend the 5′-end of retina *ALDH1* cDNA. Briefly, 2 μg of poly(A)$^+$ RNA from day 7 chick retina were reverse transcribed at 52 °C using primer 2 (Fig. 2). The RNA template was hydrolyzed, and a single-stranded AmpliFINDER anchor containing an *Eco*RI site was ligated to the 3′-end of the cDNA using T4 RNA ligase. The cDNA was amplified using the nested primer 1 and AmpliFINDER primer. RACE products were cloned in pBluescript and sequenced.

DNA Transfection Analysis—Genomic DNA from the upstream regions of the retina and liver *ALDH1* transcripts was digested with restriction enzymes to generate fragments of various lengths and ligated to the 5′-end of the chloramphenicol acetyltransferase (CAT) gene in the pCAT basic vector (Promega). The constructs tested contained the following 5′-flanking regions: −97 bp, −234 bp, −722 bp, −1184 bp, −1638 bp, −2433 bp, relative to exon 1 (first exon of the retina *ALDH1* transcript), and −1.3 kb and −4 kb, relative to exon 3 (first exon of the liver *ALDH1* transcript). The 3′-ends of the genomic constructs corresponded to either a *Bst*YI site located at position +68 in exon 1 or a *Bgl*II site located at position +146 relative to the start site of the liver *ALDH1* transcript. To prepare primary retinal cultures, day 5 retinas were dissected using an inverted microscope and mechanically disrupted. The equivalent of four retinas/100-mm culture dish were plated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and penicillin/streptomycin. Primary liver cultures were prepared by dissecting livers from embryos at either day 6 or day 7 and trypsinizing the tissue before plating. Cells were transfected with 10 μg of calcium phosphate-precipitated DNA 2–3 days after plating. The DNA was removed after 16 h, and the cells were harvested 48–50 h later. CAT activity was measured using [^{14}C]chloramphenicol in the presence of *n*-butyryl coenzyme A. Hirt DNA was extracted from one-tenth of each plate harvested for the CAT assay and served as an internal control for the amount of transfected DNA (22).

DNase I Footprinting Analysis—DNA probes labeled at one end were produced by linearizing plasmids containing either a 486-bp *Xmn*I/*Bgl*II fragment (−720 to −1206 bp) or a 422-bp *Ava*II/*Nde*I fragment (−1214 to −1636 bp) with either *Hin*dIII (top strand) or *Xba*I (bottom strand) and filling in with Klenow polymerase in the presence of [α-^{32}P]dCTP. Labeled DNA fragments were released from the vector by digesting with either *Xba*I (top strand) or *Hin*dIII (bottom strand) and purified by acrylamide gel electrophoresis and electroelution. Nuclear extracts were prepared from day 7 retina and day 15 liver as described (23).

DNase I footprinting was carried out as described except that polyvinyl alcohol was omitted from the binding buffer (24). Briefly, the radiolabeled DNA probe (10 fmol) was incubated with the indicated nuclear extracts in binding buffer. An equal volume of 5 mM $CaCl_2$, 10 mM $MgCl_2$ was added, followed by DNase I (DPFF code; Worthington) to a concentration of 0.25–0.4 μg/ml. The reaction was stopped after 1 min, and the DNA was purified by phenol/chloroform extraction and ethanol precipitation. Samples were resuspended in formamide loading buffer and denatured at 90 °C for 3 min prior to electrophoresis through an 8% polyacrylamide denaturing gel.

RESULTS

Identification of the Transcription Start Site of the Retina and Liver ALDH1 mRNA—As shown in Fig. 1, *ALDH1* mRNA is considerably more abundant in day 5 chick retina than in d19 retina. In contrast, levels of *ALDH1* mRNA increase with differentiation in the liver. There is a difference in the migration rates of the retina and liver *ALDH1* mRNAs, with the retinal transcript migrating at a slower rate than the liver transcript (compare *lanes 2* and *3* in Fig. 1).

To determine the basis for the size difference between retina and liver *ALDH1* transcripts, we screened a day 7 chick retina cDNA library and an adult chicken liver cDNA library with a previously characterized 1.5-kb *ALDH1* cDNA obtained from a chick retina library (3). We then sequenced the ends of the longest *ALDH1* cDNAs obtained from both libraries. As shown in Fig. 2, there is divergence in the retina and liver *ALDH1* cDNA sequences 32 nt upstream of the translation start codon. The retina *ALDH1* cDNA (start site indicated by the *arrowhead*) has an additional 271 bp of 5′-untranslated sequence compared with liver *ALDH1* cDNA (start site indicated by the *second arrowhead*). The 5′-most 33 bp of liver *ALDH1* cDNA is unique to the liver transcript. Consequently, the predicted

amino acid sequence of ALDH1 is identical for both the liver and retina protein; however, the 5′-untranslated sequences of the two transcripts are different with the exception of the 32 nucleotides immediately upstream of the start codon. These results suggest different mechanisms for transcriptional regulation of the *ALDH1* gene in liver and retina.

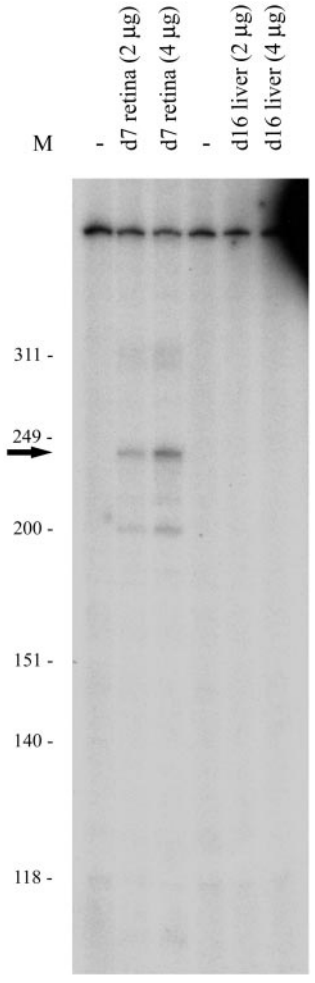

FIG. 4. **S1 nuclease mapping of the 5′-end of retina *ALDH1* mRNA.** Two or four μg of poly(A)$^+$ RNA from day 7 (*d7*) retina or day 16 (*d16*) liver, with tRNA as a negative control (−), were hybridized to a *Bgl*II-*Sty*I* fragment labeled at the *Sty*I site with [γ-^{32}P]ATP and polynucleotide kinase. The location of the *Sty*I site is indicated in Fig. 2. The uppermost positive band is indicated by an *arrow*. End-labeled φX174 phage DNA digested with *Hin*fI served as the size marker.

We used a combination of primer extension analysis, S1 nuclease analysis, and sequencing of RACE products to more accurately define the transcription start site of the retina *ALDH1* (*ALDH1-R*) transcript. Two primers were used for primer extension analysis, primers 1 and 2 (indicated in Fig. 2). Primer 2 generated a product of ~180 nucleotides, while primer 1 generated products between 72 and 74 nucleotides (Fig. 3). Primer extension products were detected using poly(A)$^+$ RNA from day 7 retina but not from day 12 liver. The predicted transcription start sites of the *ALDH1-R* mRNA obtained with these two primers are in agreement, located within 2–4 nucleotides of each other (Fig. 2). The primer extension analysis suggests that the *ALDH1-R* transcript extends an additional 37 nucleotides beyond the longest cDNA obtained by library screening.

For S1 nuclease analysis, we used a *Bgl*II/*Sty*I genomic DNA fragment labeled at the *Sty*I end located 245 bp downstream of the longest primer extension product. This DNA fragment was annealed to day 7 retina poly(A)$^+$ RNA and day 16 liver poly(A)$^+$ RNA, digested with S1 nuclease and run on a denaturing polyacrylamide gel. The S1 nuclease product obtained with this probe was ~235–240 nt long, 5–10 nt downstream of the primer extension products (Figs. 2 and 4). This product was found exclusively in the day 7 retina lanes.

RACE products were generated by reverse transcribing day 7 retina poly(A)$^+$ RNA using primer 1, followed by polymerase chain reaction amplification and cloning of the cDNA. Fifteen different cDNA products were sequenced. One of the RACE products had a 5′-end that extended as far as the S1 nuclease and primer extension products (Fig. 2). Seven of 15 RACE products had 5′-ends 15–27 bp downstream of the longest RACE product. Together, these results indicate that the *ALDH1-R* transcript has a 5′-untranslated region of ~373 nt.

Next, we identified the transcription start site of the liver *ALDH1* (*ALDH1-L*) transcript by primer extension. Poly(A)$^+$ RNA from day 12 liver was annealed to primer 3 (indicated in Fig. 2), and cDNA was generated by reverse transcription. A primer extension product of ~145 nt was obtained (data not shown), indicating that the *ALDH1-L* transcript is ~15 nucleotides longer than that predicted based on the sequence of the longest cDNA obtained from the liver cDNA library. *ALDH1-L* mRNA therefore has a predicted 5′-untranslated region of ~80 nt. In comparison, human *ALDH1* mRNA obtained from adult liver has 53 nt of 5′-untranslated sequence based on primer extension analysis (25).

Structure of the ALDH1 Gene—A chicken genomic library was screened with the 5′-ends of retina and liver *ALDH1* cDNAs, respectively. Positive bacteriophage were isolated, and

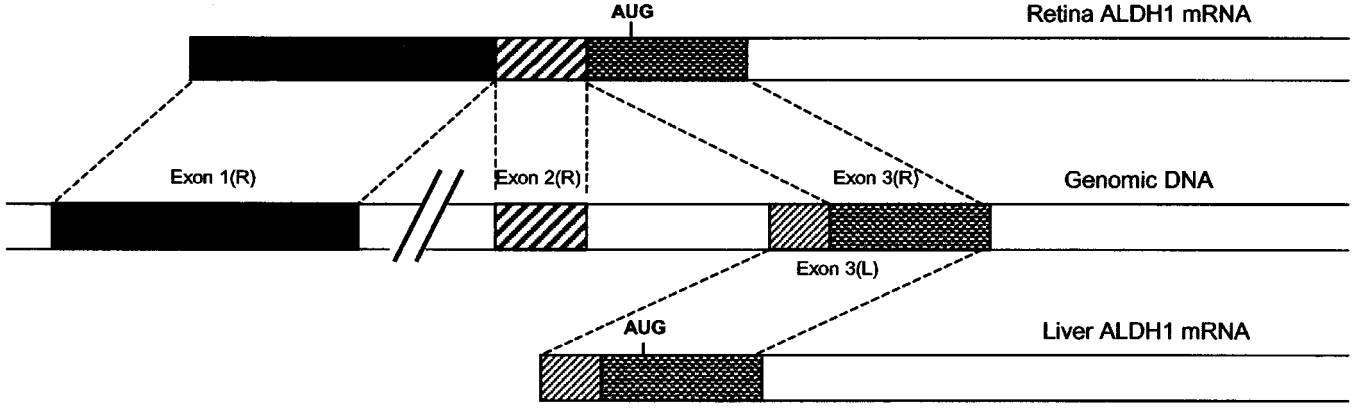

FIG. 5. **Structure of the 5′-end of *ALDH1*.** The first 3 exons of the chicken *ALDH1* gene in relation to retina (*R*) and liver (*L*) *ALDH1* mRNAs are indicated. Liver *ALDH1* mRNA initiates within intron 2, 48 nt upstream of exon 3(R). The gap between exons 1 and 2 is indicated by the *parallel bars*.

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Hu    AAATTCGATG CTGGAGCACT GGTTTCTTAA GGATTTAAGT TTAAAGTCAA AGGCTTCCTG CCCTAGGTGT TACAAATAAG TAGTGTCGTT TTCTTTTTTT
Ch    CCTGG-T--- -A-ATTGTT- CAGCAGAA-- TTTA-ATCAA GCTG-A-G-- -A-A--GTGA -TT-T---C- --AG------ --------•• --T-CCC---

                                                                                                                  *
Hu    GCTCTGAGTT TGTTCATCCA ATCGTATCCG AGTATGCAAA TAAACTTTAG CCCGTGCAGA TAAAAA•AGG AACAAATAAA GCCAAGTGCT CTATCAGAAC
Ch    --AGG--A-- CT-------- ---A---T-T T--------- --T--CG--- -TG--A---- ------AG-- C--------- --T---G--- -AGG---G--
                                                                                                                  *

Hu    CAAA•TTGCT GAGCCAGTCA CCTGTGTTCC AGGAGCCGAA TCAGAAATGT
Ch    A---A----- -C--TTCC-T GA-ACC-CAT TTC-C-T-TG -TTC-GGA-C GTACTCAAAC TCCTCACCTG GCAACATGA
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FIG. 6. **Comparison of the upstream regions of the chicken and human liver *ALDH1* mRNAs.** Identical residues are indicated by a *dash*. The *closed circles* denote the absence of a residue. The *asterisks* indicate the transcription start sites of human (*Hu*) and chicken (*Ch*) liver *ALDH1*. The CCAAT, octamer, and TATA/GATA boxes are indicated in *boldface type*. The methionine start codons are *underlined*.

a restriction enzyme map of the 5′-end of *ALDH1* genomic DNA was generated. Genomic DNA fragments containing exons were subcloned and sequenced. Genomic DNA analysis demonstrated that the *ALDH1* gene expressed in retina has two additional upstream exons (exon 1-retina (R) and exon 2-R) in comparison with that expressed in liver (Fig. 5). The translation initiation codon is identical for both retina and liver ALDH1 and is located within the third exon of *ALDH1*. The *ALDH1-L* transcript initiates 48 bp upstream of exon3-R, resulting in an extended exon 3-liver (L) sequence (Fig. 5). The second exon of *ALDH1* is located ~290 bp upstream of the third exon and is 72 bp in length.

We were unable to identify a bacteriophage that contained DNA sequences that hybridized to both exon 1 and exon 2 of *ALDH1*, suggesting a large gap between these two exons. We therefore carried out pulse field gel electrophoresis to determine the size of the gap. Chicken genomic DNA was cut with restriction enzymes generating large size fragments, and replicate blots were hybridized to DNA fragments corresponding to exon 1 and exons 2/3, respectively. These two probes hybridized to a number of similar size fragments, all of which were >50 kb with the exception of a ~30–35-kb *Bam*HI fragment (data not shown). Since there are no *Bam*HI sites within at least 6 kb of DNA flanking the 5′-end of exon 1 and the 3′-end of exon 2, we estimate that the gap between these two exons is a maximum of 24–29 kb.

We compared the sequence upstream of the chicken liver *ALDH1* transcription start site with that of human genomic *ALDH1* (accession number J04748) (25) and found significant areas of similarity between these two promoter regions. There were 112/148 identical residues in the region encompassing +18 to −130 bp of human *ALDH1* (Fig. 6). Human *ALDH1* has a presumptive TATA box (ATAAAA) with an overlapping GATA box (AGATAA) located 32 bp upstream of the transcription start site. An identical motif was found 36 bp upstream of the chicken liver *ALDH1* transcription start site. Similarly, the human and chicken *ALDH1* shared a CCAAT box at −74 bp and −78 bp, respectively. Additional putative regulatory elements included an octamer binding site (ATTTGCAT; opposite strand) between the putative TATA and CCAAT boxes. Similar octamer motifs located upstream of the TATA box have been shown to bind to the ubiquitous transcription factor OTF-1 (26). The translation start codon of chicken ALDH1 is located 30 bp downstream of the human ALDH1 start codon.

Transcriptional Activity of the Promoter Regions of the ALDH1 Gene Expressed in Retina and Liver—We generated a series of pCAT reporter constructs containing 97, 234, 722, 1184, 1638, and 2433 bp of 5′ genomic DNA flanking exon 1 as well as two constructs containing either 1.3 or 4 kb of 5′ genomic DNA flanking exon 3(L). These constructs were transiently transfected into primary cultures of day 5 retinal cells and day 6/7 liver cells, both of which express elevated *ALDH1* mRNA. The cells were transfected within 2–3 days of plating. CAT activity was measured using Hirt DNA as an internal control for the amount of transfected DNA.

As shown in Fig. 7, 97 bp of 5′-flanking DNA generated an 11-fold increase in CAT activity in retinal cultures compared with a pCAT basic construct without promoter or enhancer. CAT activity peaked with the −234 bp construct, producing on the average a 24-fold increase over pCAT basic. Similar levels of CAT activity were obtained with the −722 and −1184 bp constructs. Additional 5′-flanking sequences, to −1638 bp and −2433 bp, generated a 2-fold decrease in CAT activity. Increases in CAT activity, of 8- and 4-fold, were also observed with the −1.3 kb (L) and -4 kb (L) *ALDH1* constructs, respectively. In comparison, a pCAT construct containing the SV40 promoter and enhancer generated a 50–100-fold increase in CAT activity (data not shown).

Transfection of liver cultures with the same constructs yielded a different pattern from that observed in the retinal cultures. First, the −4 kb (L) *ALDH1* construct generated a higher level of CAT activity in liver cultures, 11-fold compared with 4-fold for the retina cultures. Second, although increasing levels of CAT activity were observed with the −97, −234, and −722 bp *ALDH1* constructs, peaking at 12.5-fold with the latter construct, overall activity was reduced compared with that observed in retinal cultures. Most striking, however, was the dramatic decrease in CAT activity observed with the −1184 bp construct (to 4-fold) and −1638 and −2433 bp constructs (to background levels). These results suggest the presence of inhibitory elements between −722 and −1638 bp. Although a decrease in CAT activity was also observed upon transfection of the −1638 and −2433 bp constructs in retinal cultures, the decrease was much less dramatic (2-fold) than in liver. Similar to retinal cultures, transfection of the pSV40CAT construct in liver cultures generated a 47–100-fold increase in CAT activity (data not shown).

Footprinting of the −720 to −1636 bp Region—Transfection of liver cultures with the −722 bp construct resulted in a 12.5-fold increase in CAT activity; in contrast, only basal CAT activity was observed with the −1638 and −2433 bp constructs. DNase I footprinting analysis was carried out to identify transcription factors and DNA binding elements that might be involved in the repression of *ALDH1* transcription in liver. As shown in Figs. 8 and 9*A*, four footprints were identified in the −720 to −1636 bp region. None of the footprints involved an extended CTTT/CCTT tetranucleotide repeat located at −891 to −1022 bp (Fig. 9*A*). Footprint I at position −800 bp is shared between liver and retina. Footprint II is unique to the liver, and footprint IV is unique to the retina. Although shared between retina and liver, the footprint III pattern differs between these two tissues, with the retina footprint extending from −1338 to −1356 bp and the liver footprint extending from −1339 to −1361 bp. The four footprints were observed on both the top (Fig. 8) and bottom strands (data not shown).

Putative transcription factor binding sites in all four foot-

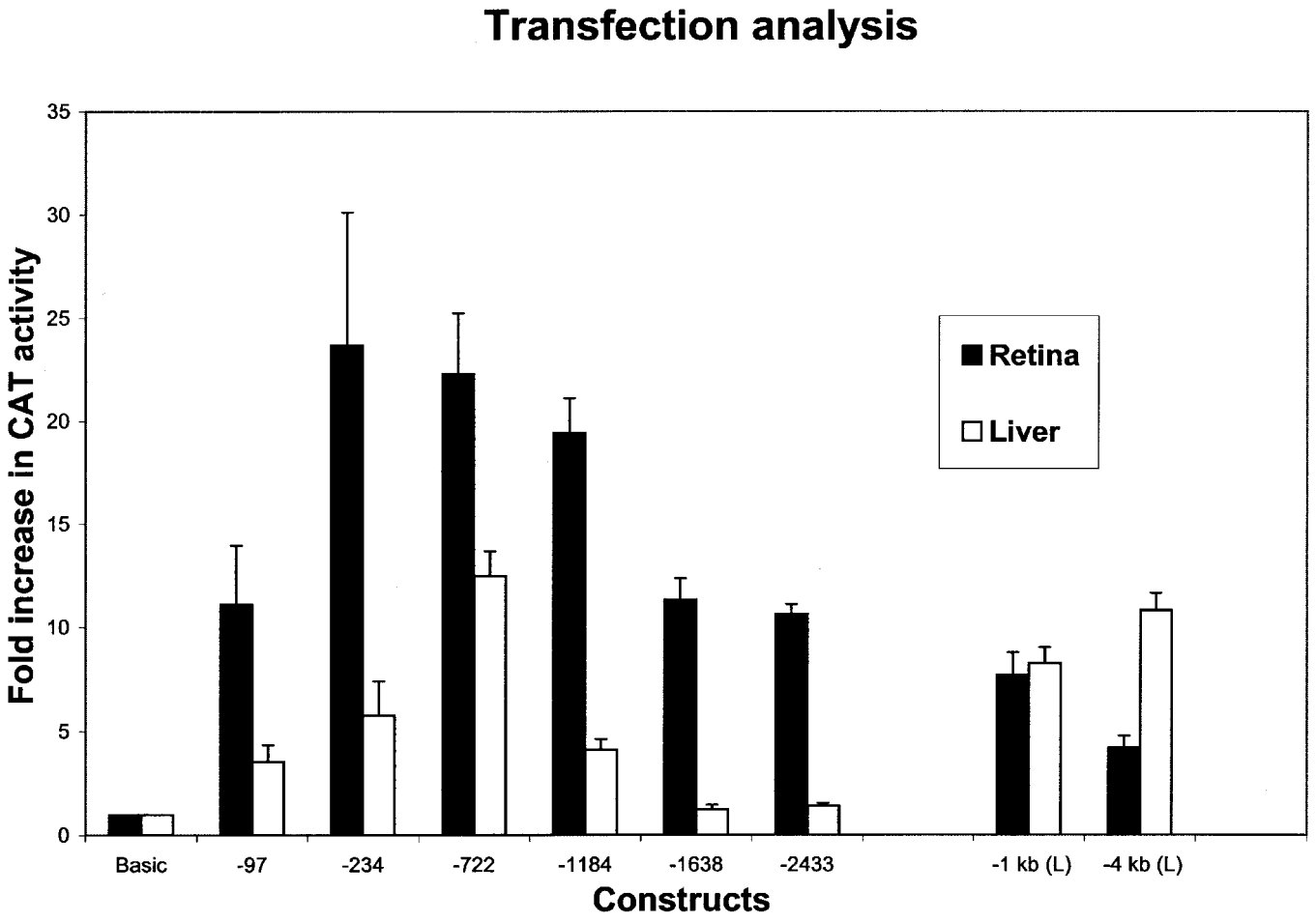

FIG. 7. **Analysis of *ALDH1* regulatory activity.** The pCAT deletion constructs were obtained by digesting *ALDH1* genomic DNA with restriction enzymes that cut at the following sites: −97, −234, −722, −1184, −1638, and −2433 bp, relative to exon 1, and −1 and −4 kb, relative to exon 3(L). Primary cultures of day 5 retina (*shaded bars*) or day 6/7 liver (*open bars*) were transfected with each of the constructs. CAT activity is reported as -fold increase over the promoterless parent vector pCAT basic. Hirt DNA served as the internal standard for transfection efficiency. The results shown are an average of 4–6 independent experiments with S.E. indicated by the *error bars*.

prints were identified using the GeneTool and IFTI programs (Fig. 9*B*). Footprint I, common to retina and liver nuclear extracts, contains an HNF-6-like consensus binding site, (G/A/T)(C/A/T)(A/T)ATTGA(T/C)T(A/T)(A/T)(G/A/T) (27). Liver-specific footprint II contains a well conserved NF-κB binding site (consensus GGG(A/G)(A/C/T)T(C/T)(C/T)(A/C/T)C) (28). Retina-specific footprint IV includes a consensus immunoglobulin heptamer element and a consensus metal regulatory element (29, 30). Although shared by retina and liver, footprint III is of special interest because the protected residues differ between the two tissues. The only consensus binding site that could be identified in this region is a Ubx-like element located on the bottom strand (consensus binding site TTAAT(G/T)(A/G)CC) (31) and an AP1 half-site GTCAT (32). It is not clear whether the Ubx consensus site is present in the retina footprint III, since it is located at the 5′-most end of the retina footprint.

DISCUSSION

ALDH1 has been proposed to be involved in the formation of an RA gradient in the developing retina. In contrast, the primary role for ALDH1 in the liver is believed to be in the detoxification of reactive aldehydes. A consequence of such divergent roles is that ALDH1 is enriched in the undifferentiated dorsal retina, whereas its levels increase with tissue maturation in the liver. Special regulatory mechanisms must have evolved to ensure correct spatial and temporal distribution of ALDH1 during development. This study addresses the regulation of the *ALDH1* gene in the developing chick retina and liver. Structural analysis of the *ALDH1* gene revealed two 5′-noncoding exons specifically transcribed in the developing retina. As a result, the sequences flanking the retina and liver transcription start sites are completely different, suggesting that *ALDH1* transcription in these two tissues must be under the control of different regulatory elements. Alternative 5′ exons have been identified in a number of other genes, including the human *PAX-6* gene and the Wiskott-Aldrich syndrome gene (33, 34). In both these cases, promoter analysis of the upstream regions of the two alternative 5′ exons has suggested the presence of cell-specific regulatory elements. The glucocorticoid receptor gene, with 11 different exon 1 sequences, represents a complex example of differential promoter usage, with a number of glucocorticoid receptor transcripts shown to have tissue-specific first exons (35).

The chicken *ALDH1* gene described here represents one of the most clear cut examples of differential usage of 5′ exons in different cell types. The *ALDH1* gene is unusual in that, rather than alternative first exons, the *ALDH1-L* transcript initiates within intron 2 and shares exon 3 with the *ALDH1-R* transcript. As a result, at least part of the regulatory elements of the *ALDH1* gene expressed in liver reside within intron 2. Sequence comparison of the 290-bp intron 2 with the upstream region of the published human *ALDH1* gene reveals a high level of similarity, with 112/148 identical residues in the region immediately upstream of the liver *ALDH1* transcription start site. There are consensus TATA/GATA, CCAAT, and octamer

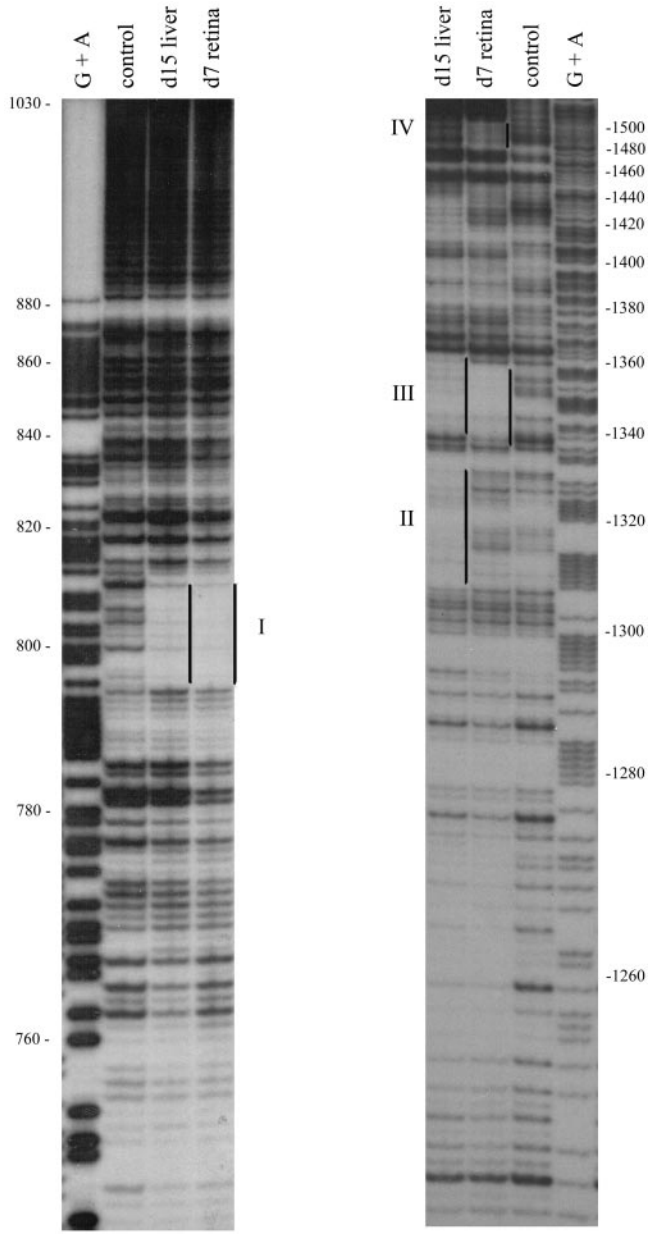

FIG. 8. **DNase I footprinting of the −720 to −1636 bp region upstream of *ALDH1* exon 1.** Two DNA fragments spanning either the −720 to −1208 bp region or the −1214 to −1636 bp region were labeled at one end of the coding strand, incubated with day 7 (*d7*) retina (15 μg) or day 15 (*d15*) liver (30 μg) nuclear extracts, digested with DNase I, and run in an 8% denaturing polyacrylamide gel. No nuclear extract was added to the lanes marked *control*. Footprints I–IV are indicated by the *vertical lines* and *numbered* on the *side*. The tetranucleotide repeat (−891 to −1022 bp) can be visualized at the *top* of the *gel* on the *left*. The *G + A lanes* represent the purine sequence of probe DNAs. Markers indicated on the *side* refer to positions relative to the transcription start site of retina *ALDH1* mRNA.

boxes present in both the chicken and human sequences (25, 36). Yanagawa *et al*. (36) have defined a minimal promoter region from −91 to +53 bp of human "liver" *ALDH1*. Using the CAT reporter gene, they observed a 65-fold increase in CAT activity in the hepatoma cell line Hep3B compared with the promoterless pCAT basic vector. In contrast, only 6–7-fold increases in CAT activity were observed in LTK and K562 cells. These results suggest that the minimal promoter region is sufficient to direct at least some level of tissue-specific expression. There is a putative RA response element-like sequence (TGTTCA rather than the consensus AGTTCA half-site) immediately upstream of the CCAAT box in the human *ALDH1* sequence, leading to the suggestion that RARα may be regulating *ALDH1* transcriptional activity through this site (37). This RA response element-like sequence is not conserved in chicken, suggesting that either chicken *ALDH1* is not regulated by RA or the chicken RA response element is located in a different region.

Our DNA transfection analysis suggests tissue-specific components governing the transcriptional activity of the *ALDH1* gene expressed in liver and retina. In general, the sequences flanking the *ALDH1-L* transcription start site are more active in liver cells, while the sequences flanking the *ALDH1-R* transcription start site are more active in retinal cells. However, the most dramatic difference between retina and liver cells was observed with *ALDH1* constructs containing >722 bp of DNA flanking the 5′-end of exon 1. CAT activity was reduced 3-fold in liver cells transfected with the −1184 bp construct, in contrast to retinal cells, which showed no decrease in CAT activity. Longer constructs generated even more striking differences between these two tissues, with only background CAT activity observed in liver cells transfected with the −1638 and −2433 bp constructs. These results suggest the presence of tissue-specific inhibitory elements located 722–1638 bp upstream of the *ALDH1-R* transcription start site. The most notable feature of this region is a CTTT/CCTT tetranucleotide sequence repeated 33 times, located from −891 to −1022 bp. A number of tetranucleotide repeats have been identified either within genes or within their flanking regions, including the *RB1* and neurotensin receptor genes (38, 39). For the most part, the roles of these repeats remain poorly defined. Recently, however, a tetranucleotide repeat located in the first intron of the tyrosine hydroxylase gene has been shown to have transcription regulatory activity *in vitro* (40). Ten copies of this repeat increased basal transcription up to 9-fold, and gel shift assays indicated that these repeats form complexes with factors present in HeLa nuclear extracts. The authors have suggested that tetrarepeat sequences of the TCAT type constitute a new class of regulatory elements. Our DNase I footprinting analysis provides no evidence of nuclear proteins interacting with the CTTT/CCTT repeats. However, it is still possible that the presence of this repeat may modify the structure of the *ALDH1* gene in such a way as to either enhance (in dorsal retina) or inhibit (in liver) transcription activity of factors involved in *ALDH1* regulation.

Four footprints have been identified in the DNA neighboring the tetranucleotide repeat. One of these footprints, footprint II, is liver-specific and may represent protein-DNA interaction critical for inhibition of "retinal" *ALDH1* promoter activity in non-dorsal-retinal cells such as liver. The liver-specific footprint has a well conserved 10-bp consensus NF-κB binding element. The NF-κB family of transcription factors, including NF-κB1, RelA, c-Rel, RelB, and NF-κB2, regulates genes involved in cell proliferation, cell differentiation, and immune and inflammatory responses (41). NF-κB/Rel transcription factors can be found in both inactive cytoplasmic (when bound to IκB proteins) and active nuclear forms (42, 43). Although primarily believed to function as a transcriptional activator, nuclear NF-κB/Rel can also function as a negative regulator of transcription. For example, NF-κB/Rel has recently been implicated in the silencing of the human ζ-globin gene (44). We propose that an NF-κB/Rel-like factor could also play a role in the transcriptional repression of *ALDH1* from the "retinal" promoter in liver cells. Binding of this factor and recruitment of other factors to this area could result in the alteration in footprint III observed in liver.

At this time, we can only speculate as to whether NF-κB/Rel factors could also play a role in the repression of *ALDH1*

A

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                                                                                                  Footprint
TCTGGGTATG GAGAGAGGAG AGCCCAAGCC TCAGAAATGG GGGATTGTTA GTTAGGTCTT CTCCTGGCAC TAGAGCCTAA ACACTACATT ACCTGCACTG      -1592
  IV
TGCACACACC TGAAACCTGG TCTGTTAAAT TTGTGTGCTG CTGTTTTATT AAGCACTGTA TTACCTAACC CAACAAATAA TCTAAAGGTA GTTTTGAAGT      -1492
                                        Footprint III                        Footprint II
GAGGTCAGAG TCAGCTCAGC ACTGGATGTA TTTAAGGTCA TTAAAGATTC AGTGTCATGG TTTTGCATGG GAATCTTCCA AAGAAGCTCT GTCAAAAAGC      -1392

AGTTATTCAA AAAGCTGTTA TAATACATTC AATATAAACA TGTCTATGCA CAAAATAAAT TATAAGAAGA AAATTCATAT GTAAAGATCT TTGTGCATAA      -1292

GCATAAAATC TAGAGATTAT TCAAGGTTCA GAAAGTAGTA CATTGCCTTT ACACTGGGAT CATTCTCTTC TACCTACTTT GTGTGTTTCT AATTCCTAGC      -1192

ACAAGGAAAA CTACATTTAA TTTTGTGTAA TACAGACTAA CAAAACCTGA TGTTTCCCTT GCTTTTTTCT TTTCCTTTCT TTCTTTCTTT CTTTCTTTCT      -1092

TTCTTTCTTT CTTTCTTTCT TTCTTTCTTT CTTTCTTTCT TTCTTTCTTT CTTTCCTTCT TTCCTTCTTT CCTTCTTTCC TTCTTTCCTT CTTTCCTTTC      -992
                                                                                    Footprint I
CTTCTTTTTA TTTTTTTTAA GCTAAGGAAG AAGAAGAGGA ACAAACCACT TTCTTCCATG AGTATTTTGC TGACAGAAAT ACTTAAATCA ATAAATTGTG      -892

GAAAAAGCTG TTGATAGCGT TGCAACAGTT GTATTTTGCA ATTTGAGCTG TTCTCCATTG GGTGCTGAAG TATTTCCGAA CAAAAAGCAT AAGTCTGAGT      -792

TCAGTACAGC ATTGGATTGA GTATATTTCT TGCATGTTCC AGCAGGGAAT TGTTGTCTTT TCTTGGAAAT TAAAGGCAAA TCCATTCAGA GGGCTCTCTT      -692

CTCACCACTT TGTAGCGTAG CAGTATCCGC ACTTCCTCTC CCTCCCCCAT TTTTCTTTTC CTTTCTCCAT CCCCTTTAAA TTGTAATTCA ATCTCTTCCA      -592

ATTCATGATC TCTTTTCTCT TTTTGCACTC CCTCCACCCT CTCTCTCTCT CATTAGTTTA ACTTGAGTTA ATTAGGAATA GTAGGCAGCC CTCAATCTGT      -492

TATGTGTTAA AATAGAACAA TCCAAAACAG AAAGACACAG ACACAACTAT GTGATTTCAA TTGTCTAAAG GAGACAGAAA ACCAGTGGGG ATTCTCTTAT      -392

CAAGGCTAAC ATCTCCCCAA TGGTTAATGT AACTGTCTTT AAAATGTTCC ACAAATGCAG ATCTAGGATA ACAATCTTTC CGTCATGTTT CCTTTTCTTT      -292

TTCTTTTCTT TTCTTTTTTT TTTTTTTTTT CATTTCATCA TTGGCAAGAA TGAGTGAATT GGATTGACAT GGAGGTGAGA GAGGAAGAGA AACAGTCTAC      -192

ACGTCAAAAT GACAATGTAT ATTACATGGT TAACAGAATG AGTGGTACGT GCAATGGCTG CGGGTATTTT CATCTGCTGT GTGTACAATA AG +1CTCTT       -92
```

B

Footprints	Tissue	Location	Sequence (top strand)	Consensus binding sites
I	R,L	-812 to -795	ACT**TAAATCAATAAAT**TG	HNF-6-like
II	L	-1330 to -1308	TTGCAT**GGGAATCTTC**CAAAGAA	NF-κB
III	R	-1356 to -1338	GTCATTAAAGATTCAGTGT	
	L	-1361 to -1339	TTAA**GGTCATTAA**AGATTCAGTG	Ubx*
IV	R	-1502 to -1482	ACCTG**CACTGTG**CACACACCT	Ighept
			ACCTGCACTGT**GCACAC**ACCT	MREe

*The Ubx consensus site is on the opposite strand

FIG. 9. **Sequence analysis of footprints I–IV.** *A*, sequence of the −1 to −1592 bp region upstream of exon 1. The four footprints identified in the −720 to −1636 bp region are shown in *boldface type*, and the transcription start site of retina *ALDH1* mRNA is indicated by +1. *B*, consensus binding sites mapping to footprints I–IV. The tissue specificity (retina (*R*), liver (*L*)) of the footprints is indicated as well as their position relative to the transcription start site of retina *ALDH1* mRNA. Consensus binding sites identified using either the GeneTool or IFTI programs are indicated in *boldface type*.

expression in the ventral retina. It is of interest that the *Drosophila* NF-κB/Rel factor Dorsal has been shown to be directly involved in the specification of embryonic dorsal/ventral polarity (reviewed in Ref. 45). Although intrinsically a transcriptional activator, Dorsal can function as a repressor by recruiting transcription repressors to negative response elements. Future work will involve studying the distribution pattern of NF-κB/Rel proteins in the developing chick retina and determining whether nuclear extracts prepared specifically from ventral retina can generate footprint II. Other transcription factors that could also be involved in *ALDH1* regulation in the retina include Vax2 and Pax2, specifically expressed in the ventral retina (46–48), and Tbx5 and Tbx12, specifically expressed in the dorsal retina (49–51). The binding sites for these transcription factors remain to be characterized.

In summary, we have discovered that the *ALDH1* gene expressed in the chick dorsal retina has two additional 5′ noncoding exons, which result in a transcript that is ~300 nt longer than that found in the liver. As a result, the promoter region of the retina *ALDH1* transcript is completely different from that of the liver *ALDH1* transcript, suggesting different mechanisms for the regulation of *ALDH1* expression in these

two tissues. Analysis of the sequences upstream of the retina and liver *ALDH1* transcription start sites by transient transfections using the CAT reporter gene and by DNase I footprinting indicates that tissue-specific regulation of the *ALDH1* gene is governed by a complex array of positive and negative regulatory elements. Of note, the presence of a NF-κB binding site located within a liver-specific footprint upstream of the retina *ALDH1* transcription start site suggests a possible role for this family of transcription factors in repression of the retina *ALDH1* promoter in liver.

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REFERENCES

1. Lee, M. O., Manthey, C. L., and Sladek, N. E. (1991) *Biochem. Pharmacol.* **42,** 1279–1285
2. Lindahl, R. (1992) *Crit. Rev. Biochem. Mol. Biol.* **27,** 283–335
3. Godbout, R. (1992) *Exp. Eye Res.* **54,** 297–305
4. Chen, J., Yanagawa, Y., and Yoshida, A. (1996) *Biochem. Genet.* **34,** 109–116
5. Stewart, M. J., Malek, K., and Crabb, D. W. (1996) *J. Invest. Med.* **44,** 42–46
6. Godbout, R., Packer, M., Poppema, S., and Dabbagh, L. (1996) *Dev. Dyn.* **205,** 319–331
7. McCaffery, P., Tempst, P., Lara, G., and Dräger, U. C. (1991) *Development* **112,** 693–702
8. McCaffery, P., Lee, M.-O., Wagner, M. A., Sladek, N. E., and Dräger, U. C. (1992) *Development* **115,** 371–382
9. Grün, F., Hirose, Y., Kawauchi, S., Ogura, T., and Umesono, K. (2000) *J. Biol. Chem.* **275,** 41210–41218
10. Li, H., Wagner, E., McCaffery, P., Smith, D., Andreadis, A., and Dräger, U. C. (2000) *Mech. Dev.* **95,** 283–289
11. Mic, F. A., Molotkov, A., Fan, X., Cuenca, A. E., and Duester, G. (2000) *Mech. Dev.* **97,** 227–230
12. McCaffery, P., Posch, K. C., Napoli, J. L., Gudas, L., and Dräger, U. C. (1993) *Dev. Biol.* **158,** 390–399
13. Marsh-Armstrong, N., McCaffery, P., Gilbert, W., Dowling, J. E., and Dräger, U. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7286–7290
14. Hyatt, G. A., Schmitt, E. A., Marsh-Armstrong, N. R., and Dowling, J. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8293–8297
15. Hyatt, G. A., Schmitt, E. A., Marsh-Armstrong, N., McCaffery, P., Dräger, U. C., and Dowling, J. E. (1996) *Development* **122,** 195–204
16. Sperry, R. W. (1963) *Proc. Natl. Acad. Sci. U. S. A.* **50,** 703–710
17. Baier, H., and Bonhoeffer, F. (1992) *Science* **255,** 472–475
18. Marcus, R. C., Gale, N. W., Morrison, M. E., Mason, C. A., and Yancopoulos, G. D. (1996) *Dev. Biol.* **180,** 786–789
19. Connor, R. J., Menzel, P., and Pasquale, E. B. (1998) *Dev. Biol.* **193,** 21–35
20. Belikov, S., and Wieslander, L. (1995) *Nucleic Acids Res.* **23,** 310
21. Favaloro, J., Treisman, R., and Kamen, R. (1980) *Methods Enzymol.* **65,** 718–745
22. Hirt, B. (1967) *J. Mol. Biol.* **26,** 365–369
23. Gorski, K. M., Carneiro, M., and Schibler, U. (1986) *Cell* **47,** 767–776
24. Jones, K. A., Yamamoto, K. R., and Tjian, R. (1985) *Cell* **42,** 559–572
25. Hsu, L. C., Chang, W.-C., and Yoshida, A. (1989) *Genomics* **5,** 857–865
26. Fletcher, C., Heintz, N., and Roeder, R. G. (1987) *Cell* **51,** 773–781
27. Samadani, U., and Costa, R. H. (1996) *Mol. Cell. Biol.* **16,** 6273–6284
28. Lenardo, M. J., and Baltimore, D. (1989) *Cell* **58,** 227–229
29. Tonegawa, S. (1983) *Nature* **302,** 575–581
30. Labbé, S., Prévost, J., Remondelli, P., Leone, A., and Séguin, C. (1991*). Nucleic Acids Res.* **15,** 4225–4231
31. Ekker, S. C., von Kessler, D. P., and Beachy, P. A. (1992) *EMBO J.* **11,** 4059–4072
32. Johnsen, Ø., Murphy, P., Prydz, H., and Kolstø, A.-B. (1998) *Nucleic Acids Res.* **26,** 512–520
33. Okladnova, O., Syagailo, Y. V., Mossner, R., Riederer, P., and Lesch, K. P. (1998) *Mol. Brain Res.* **60,** 177–192
34. Hagemann, T. L., and Kwan, S. P. (1999) *Biochem. Biophys. Res. Commun.* **256,** 104–109
35. McCormick, J. A., Lyons, V., Jacobson, M. D., Noble, J., Diorio, J., Nyirenda, M., Weaver, S., Ester, W., Yau, J. L., Meaney, M. J., Seckl, J. R., and Chapman, K. E. (2000) *Mol. Endocrinol.* **14,** 506–517
36. Yanagawa, Y., Chen, J. C., Hsu, L. C., and Yoshida, A. (1995) *J. Biol. Chem.* **270,** 17521–17527
37. Elizondo, G., Corchero, J., Sterneck, E., and Gonzalez, F. J. (2000) *J. Biol. Chem.* **275,** 39747–39753
38. Liu, Y., Rasool, O., Grander, D., Lindblom, A., and Einhorn, S. (1995) *Hum. Mol. Genet.* **4,** 727–729
39. Le, F., Groshan, K., Zeng, X. P., and Richelson, E. (1997) *J. Biol. Chem.* **272,** 1315–1322
40. Meloni, R., Albanèse, V., Ravassard, P., Treilhou, F., and Mallet, J. (1998) *Hum. Mol. Genet.* **7,** 423–428
41. Baldwin, A. S., Jr. (1996) *Annu. Rev. Immunol.* **14,** 649–683
42. Pahl, H. L. (1999) *Oncogene* **18,** 6853–6866
43. Karin, M. (1999) *Oncogene* **18,** 6867–6874
44. Wang, Z., and Liebhaber, S. (1999) *EMBO J.* **18,** 2218–2228
45. Govind, S. (1999) *Oncogene* **18,** 6875–6887
46. Otteson, D. C., Shelden, E., Jones, J. M., Kameoka, J., and Hitchcock, P. F. (1998) *Dev. Biol.* **193,** 209–224
47. Schulte, D., Furukawa, T., Peters, M. A., Kozak, C. A., and Cepko, C. L. (1999) *Neuron* **24,** 541–553
48. Barbieri, A. M., Lupo, G., Bulfone, A., Andreazzoli, M., Mariani, M., Fougerousse, F., Consalez, G. G., Borsani, G., Beckmann, J. S., Barsacchi, G., Ballabio, A., and Banfi, S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10729–10734
49. Chapman, D. L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S. I., Gibson-Brown, J. J., Cebra-Thomas, J., Bollag, R. J., Silver, L. M., and Papaioannou, V. E. (1996) *Dev. Dyn.* **206,** 379–390
50. Carson, C. T., Kinzler, E. R., and Parr, B. A. (2000) *Mech. Dev.* **96,** 137–140
51. Koshiba-Takeuchi, K., Takeuchi, J. K., Matsumoto, K., Momose, T., Uno, K., Hoepker, V., Ogura, K., Takahashi, N., Nakamura, H., Yasuda, K., and Ogura, T. (2000) *Science* **287,** 134–137