Avian and Canine Aldehyde Oxidases
NOVEL INSIGHTS INTO THE BIOLOGY AND EVOLUTION OF MOLYBDO-FLAVOENZYMES*[S]

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Aldehyde oxidases are molybdo-flavoenzymes structurally related to xanthine oxidoreductase. They catalyze the oxidation of aldehydes or *N*-heterocycles of physiological, pharmacological, and toxicological relevance. Rodents are characterized by four aldehyde oxidases as follows: AOX1 and aldehyde oxidase homologs 1–3 (AOH1, AOH2, and AOH3). Humans synthesize a single functional aldehyde oxidase, AOX1. Here we define the structure and the characteristics of the aldehyde oxidase genes and proteins in chicken and dog. The avian genome contains two aldehyde oxidase genes, *AOX1* and *AOH*, mapping to chromosome 7. *AOX1* and *AOH* are structurally very similar and code for proteins whose sequence was deduced from the corresponding cDNAs. *AOX1* is the ortholog of the same gene in mammals, whereas *AOH* represents the likely ancestor of rodent *AOH1*, *AOH2*, and *AOH3*. The dog genome is endowed with two structurally conserved and active aldehyde oxidases clustering on chromosome 37. Cloning of the corresponding cDNAs and tissue distribution studies demonstrate that they are the orthologs of rodent *AOH2* and *AOH3*. The vestiges of dog *AOX1* and *AOH1* are recognizable upstream of *AOH2* and *AOH3* on the same chromosome. Comparison of the complement and the structure of the aldehyde oxidase and xanthine oxidoreductase genes in vertebrates and other animal species indicates that they evolved through a series of duplication and inactivation events. Purification of the chicken AOX1 protein to homogeneity from kidney demonstrates that the enzyme possesses retinaldehyde oxidase activity. Unlike humans and most other mammals, dog and chicken are devoid of liver aldehyde oxidase activity.

Molybdo-flavoenzymes (MOFEs)[4] constitute a small family of homodimeric oxidoreductases characterized by conserved structures (1). Until a few years ago, it was believed that the family of mammalian MOFEs consisted of only two members, *i.e.* xanthine oxidoreductase (XOR) (2–4) and the aldehyde oxidase AOX1[5] (5–8). XOR has been extensively studied and is the key enzyme in the catabolism of purines, oxidizing hypoxanthine to xanthine and xanthine to uric acid (9–14). This function is conserved throughout evolution, as the enzyme is present from bacteria to man (1). In mammals, the protein also plays an important role in milk secretion (15–17) and kidney development (18). The function of AOX1 is ill-defined, and the enzyme lacks a recognized physiological substrate. AOX1 metabolizes *N*-heterocyclic compounds and aldehydes of pharmacological and toxicological relevance (19–22). XOR and AOX1 are the products of two genes mapping on distinct chromosomes in rodents and different arms of chromosome 2 in humans (4, 7, 23, 24).

Recently, we demonstrated that the family of mammalian MOFEs is larger than originally anticipated (25–28). Mice and rats are endowed with three extra MOFEs structurally and biochemically more similar to AOX1 than to XOR. We named these proteins aldehyde oxidase homologs 1–3 (AOH1, AOH2, and AOH3). In rodents, AOH1 is synthesized predominantly in liver and lung, the only two organs that express significant amounts of AOX1 as well (28). AOH2 was originally identified in the keratinized epithelia of the stomach, esophagus, and skin (28), although the richest source of the enzyme is the Harderian gland,[6] a specialized structure present in the orbital cavity of various types of animals (29). The tissue and cell distribution of

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[4] The abbreviations used are: MOFEs, molybdo-flavoenzymes; XOR, xanthine oxidoreductase; RAL, retinaldehyde; MoCo, molybdenum cofactor; AOH, aldehyde oxidase homolog; RACE, rapid amplification of cDNA ends; RT, reverse transcription; nt, nucleotide; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; LOC, locus.

[5] The nomenclature adopted in this study is as follows. AOX1 refers to the first identified MOFE with aldehyde oxidase activity and is the product of the gene originally annotated as *AOX1* in the human and mouse sections of the NCBI data base. AOH1 and AOH2 refer to the proteins originally identified in mice as the aldehyde oxidase homologs 1 and 2 (27). In the protein section of the NCBI data base, the two proteins are also annotated as AOX3 (accession number NP_076106) and AOX4 (accession number NP_076120). AOH3 refers to the last member of the MOFE family identified (25). The protein is annotated as aldehyde oxidase 3-like 1 (Aox3l1, accession number NP_001008419) in the protein section of the NCBI database. Aldehyde oxidase is used as a general term and refers to any member of the MOFE subgroup.

[6] M. Terao and E. Garattini, unpublished observations.

AOH3 is also peculiar, as the enzyme is selectively expressed in nasal mucosa (25). Given the recent identification of AOH1, AOH2, and AOH3, the corresponding physiological substrates and homeostatic roles are unknown.

The mouse *AOX1*, *AOH1*, *AOH2*, and *AOH3* genes have strictly conserved exon structures and cluster in a small chromosomal region (aldehyde oxidase gene cluster) (25, 27). A similar arrangement of the four orthologous genes is present in rat (25). A striking conservation of exon structure is also evident when the *AOX1*, *AOH1*, *AOH2*, and *AOH3* genes are compared with the mouse and rat XOR orthologs (1, 25, 27). Based on these as well as other observations, we proposed that all *MOFE* genes arose through one or more duplication events from a single ancestor with structural similarity to *XOR* (1). Duplications of *MOFE* genes are not a peculiarity of rodents, as they are also observed in plants and insects (30–32).

The availability of the complete sequence of an ever increasing number of genomes provides a unique opportunity to determine the number and the structure of *MOFE* genes in different animal species. In this study, we describe the cloning and sequencing of the avian and canine cDNAs encoding aldehyde oxidase and paralogous proteins. In addition, we reconstruct the exon structures of *MOFE* genes in the sequenced genomes of other vertebrates. Purification of chicken AOX1 demonstrates that the enzyme is capable of metabolizing a physiological substrate like retinaldehyde (RAL). Our results contribute to the elucidation of the biology and evolution of MOFEs.

EXPERIMENTAL PROCEDURES

Purification of Chicken Kidney AOX1 Protein, Electrophoresis, and Western Blot Analysis—Unless otherwise stated, all the purification steps were carried out at 4 °C. Male chicken kidneys (75 g) were isolated and homogenized in 3 volumes of 100 mM sodium phosphate buffer, pH 7.5, with a mechanical Turrax homogenizer. Homogenates were centrifuged at 100,000 × *g* for 45 min to obtain cytosolic extracts. Extracts were heated at 55 °C for 10 min and centrifuged at 15,000 × *g* to remove precipitated proteins. Solid ammonium sulfate was added to the supernatant (40% w/v). The precipitate was collected by centrifugation at 100,000 × *g*, resuspended in 50 mM Tris-HCl, pH 7.5, and dialyzed overnight against the same buffer. The sample was applied batchwise to benzamidine-Sepharose (Amersham Biosciences) equilibrated in 100 mM Tris-HCl, containing 100 mM NaCl, pH 7.5, and rolled overnight. After extensive washing of the phase in loading buffer, AOX1 was eluted in the same buffer containing 10 mM benzamidine. The eluate was concentrated using Centricon YM-100 ultrafiltration devices and diluted (1:10 v/v) in 100 mM Tris-HCl, pH 7.4. The material was applied to a 5:5 fast protein liquid chromatography Mono Q column (Amersham Biosciences) equilibrated in 100 mM Tris-HCl, pH 7.4. The AOX1 protein was eluted at 0.5 ml/min with a linear gradient from 0 to 1 M NaCl in 100 mM Tris-HCl, pH 7.5. The purification of AOX1 was monitored by RAL oxidizing activity and quantitative Western blot analysis (25) using an anti-bovine AOX1 antibody described previously (5), according

to a chemiluminescence-based protocol (ECL, Amersham Biosciences). The antibodies do not cross-react with bovine XOR.[7] Chemiluminescent signals corresponding to AOX1 bands were quantitated with a scanning densitometer (Hoefer Scientific Instruments, San Francisco). The total amount of AOX1 immunoreactive protein in the various experimental samples is expressed in arbitrary units and is calculated on the basis of the intensity of the Western blot signal in OD multiplied by the total volume of each purification step. One arbitrary unit of immunoreactive protein corresponds to 1.0 OD of the specific AOX1 band in each experimental sample (25). The anti-rat XOR antibodies have been described (13).

SDS-PAGE was performed following standard techniques (33). Proteins were measured according to the Bradford method with a commercially available kit (Bio-Rad).

Determination of the Chicken and Dog Cross-reactivity Profiles of Anti-bovine AOX1 Antibodies—The spectrum of cross-reactivity of the anti-bovine AOX1 antibodies against chicken, dog, and mouse MOFEs was determined on extracts of COS-7 transfected with chicken AOX1, AOH, and XOR as well as dog AOH2, AOH3, and XOR full-length cDNAs. The complete coding regions of the various cDNAs were cloned in the pCMVβ plasmid expression vector (Clontech). COS-7 cells were cultured and transfected with cationic liposomes, as described previously (27).

Determination of Retinaldehyde Oxidase Activity in Tissue Cytosolic Extracts—RAL oxidase activity was measured in chicken liver, kidney, and heart, C57/Bl and DBA/2 mouse liver, as well as dog liver and kidney (26). Organs were dissected, frozen, and stored at −80 °C until processed for the determination of RAL activity. Organs were homogenized in 3 volumes (w/v) of 10 mM potassium phosphate, pH 7.4. Samples were ultracentrifuged at 100,000 × *g* for 45 min. Supernatants were collected and passed on PD10 (Amersham Biosciences) columns to eliminate endogenous NAD$^+$. Desalted samples were incubated in 100 μl of 10 mM potassium phosphate, pH 7.4, containing all-*trans*-retinaldehyde (Sigma) for 10 min. The reaction was stopped by addition of 100 μl of *n*-butanol/methanol (95:5 v/v) containing 0.005% w/v of butylated hydroxytoluene (Sigma) and was vortexed. The organic phase was separated, and an aliquot (20 μl) was loaded onto RP-18 reverse phase HPLC columns (Waters), using a Beckman apparatus equipped with a UV-visible detector (Beckman Instruments, Palo Alto, CA). The retention times of all-*trans*-retinoic acid and all-*trans*-RAL were determined using authentic standards of the two compounds (Sigma). The amounts of retinoic acid were determined by integrating the area of the specific chromatographic peak and comparing it to an appropriate calibration curve. The enzymatic activity equivalent to the oxidation of 1 nmol of RAL to retinoic acid/min is defined as 1 unit.

Characterization of the Purified Chicken AOX1 Protein by Mass Spectrometry—MALDI-mass spectrometric and electrospray ionization tandem mass spectrometric analyses of chicken AOX1 tryptic peptides were performed according to

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[7] E. Garattini and M. Terao, unpublished results.

standard protocols following *in gel* tryptic digestion (25). Briefly, the Coomassie-stained gel slice corresponding to purified AOX1 was incubated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate at 56 °C for 30 min to reduce disulfide bridges. Thiol groups were alkylated upon reaction with 55 mM iodoacetamide in 100 mM ammonium bicarbonate at room temperature in the dark for 20 min. Tryptic digestion was carried out overnight at 37 °C in 50 mM ammonium bicarbonate and 12.5 ng/μl of trypsin (Promega, Madison, WI). Peptides were extracted twice in 50% acetonitrile, 5% formic acid. The combined extracts were lyophilized and redissolved in 0.5% formic acid and desalted using ZipTip (Millipore, Bedford, MA). Peptides were eluted in 50% acetonitrile, 0.5% formic acid. The eluate was mixed 1:1 (v/v) with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid in acetonitrile, 0.1% trifluoroacetic acid 1:3 (v/v). Mass mapping of tryptic peptides was performed with a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker, Bremen, Germany). Data generated were processed with the Mascot program (25) allowing a mass tolerance of $\leq$0.1 Da.

cDNA Cloning, Nucleotide Sequencing, and Determination of the Intron/Exon Structure of the Corresponding Genes—The chicken AOX1 cDNA was isolated as three overlapping fragments (corresponding to exons 3–17, 17–31, and 31–35) by RT-PCR from kidney RNA. The couples of oligonucleotides used as primers are as follows: 5′-CAGGAACTAAGTATGGCTGTGGAG3-′ (nt 130–153 of the chicken AOX1 cDNA); 5′-ATCTTAGCATGAGCTCTGGAACTAG-3′ (complementary to nt 1855–1879); 5′-AATGTAGAACTGAGTCAGTCTCCC-3′ (nt 1713–1736); 5′-CAACCTCTGAACAAGCAGTTCCAT-3′ (complementary to nt 3499–3522); 5′-ACGATGCAAATATGGACTGGGAGAA-3′ (nt 3442–3466); and 5′-CTGTTCAGGTCTCATGCATTCTGG-3′ (complementary to nt 4012–4035).

The chicken AOH cDNA was isolated as three overlapping fragments (corresponding to exons 3–16, 15–27, and 26–35) by RT-PCR from Harderian gland RNA. The couples of oligonucleotides used as primers are as follows: 5′-GTGGTGCATGCACTGTGATGTTGT-3′ (nt 211–234 of the chicken AOH cDNA); 5′-CTTTGTAGCACTCCCAAAGCACTC-3′ (complementary to nt 1712–1735); 5′-ACAGTGGAATGATCAGATGCTGAGT-3′ (nt 1511–1535); 5′-TGAGTGACTAGTACAGACCCATCTA-3′ (complementary to nt 3148–3172); 5′-CATGTACAGAGGAGTTAACCGGAC-3′ (nt 2909–2932); and 5′-GGATATATCAATGGCCCACGGCTT-3′ (complementary to nt 4041–4064).

The dog AOH2 cDNA was isolated as three overlapping fragments (corresponding to exons 1–15, 14–26, and 25–35) by RT-PCR from lacrimal gland RNA. The couples of oligonucleotides used as primers are as follows: 5′-GGTATGATGGCTTCTGTTCCCAAT-3′ (nt 15–38 of the dog AOH2 cDNA); 5′-TATTCAGTCCTCGCCTCACTTTGA-3′ (complementary to nt 1606–1629); 5′-CATTGTCAATGCTGGCATGAGTGT-3′ (nt 1340–1363); 5′-CCCCTCTTCTTCCAGTAGTTCTTT-3′ (complementary to nt 3005–3028); 5′-TACATAACTGCTGTGGCATCTCAG-3′ (nt 2814–2837); and 5′-GGATCAAGACACACGGATAGACCA-3′ (complementary to nt 4008–4031).

The dog AOH3 cDNA was isolated as three overlapping fragments (corresponding to exons 1–15, 14–26, and 25–34) by RT-PCR from nasal mucosa RNA. The couples of oligonucleotides used as primers are as follows: 5′-ACAATGCCTTGCCCATCGAAATCC-3′ (nt 136–159 of the dog AOH3 cDNA); 5′-CACCAGAGTCCTCTTGAATTCCAC-3′ (complementary to nt 1681–1704); 5′-AGGAAGGCACAGGCACTATTGAGG-3′ (nt 1496–1519); 5′-CCAACTGAAAACTTCATGGGGACG-3′ (complementary to nt 3177–3200); 5′-ATTTGGCTTCCCACAAGGAACCCT-3′ (nt 2916–2939); and 5′-CATCTCTGTGAACCGATCTGCACA-3′ (complementary to nt 4093–4116).

The appropriate DNA fragments were subcloned into the pCR2.1 plasmid using the TA cloning kit (Invitrogen) and sequenced according to the Sanger dideoxy chain termination method, using double-stranded DNA as template and T7 DNA polymerase (Amersham Biosciences). Oligodeoxynucleotide primers were custom synthesized by Sigma. Computer analysis of the DNA sequences was performed using the GeneWorks sequence analysis system (Intelligenetics, San Diego, CA). The nucleotide and protein sequences of the full-length chicken AOX1 and AOH, as well as dog AOH2 and AOH3 cDNAs were compared with the corresponding genomic sequences present in the NCBI public data bases. This resulted in the determination of the exon/intron structure of the corresponding genes.

Determination of the 5′ and 3′ Ends of the Chicken and Dog Transcripts—Total RNA was extracted from chicken kidney (AOX1), chicken Harderian glands (AOH), dog lacrimal glands (AOH2), and dog nasal mucosa (AOH3). The poly(A$^+$) fraction of the RNA was selected according to standard procedures (33). 5′-rapid amplification of cDNA ends (RACE) was performed with the commercially available Marathon cDNA amplification kit (Clontech), according to the nested PCR protocol included, using the primers indicated as follows: chicken AOX1 specific primer (SP1), 5′-TACTTCCAACACCTTCCACTGTGG-3′ (complementary to nt 277–300 of the cDNA), and nested primer (SP2), 5′-CTGTGGTGACTGCCATACCATACA-3′ (complementary to nt 259–282); chicken AOH SP1, 5′-CTTTGTAGCACTCCCAAAGCACTC-3′ (complementary to nt 1712–1735), and SP2, 5′-CCTGCTGACAACATGTCCTC-3′ (complementary to nt 1129–1148); dog AOH2 SP1, 5′-GTCACTGGATAGTGGTGGATCTTC3-′ (complementary to nt 221–244), and SP2, 5′-GGATAGTGGTGGATCTTCTTGGTC-3′ (complementary to nt 215–238); and dog AOH3 SP1, 5′-TCCTGTGAGGCGTAAGTTCTTTCG-3′ (complementary to nt 241–264). This 5′-RACE reaction did not require a nested protocol.

The 3′-RACE was conducted as above with the following primers: chicken AOX1 SP1, 5′-TTTGCACTGAACAGCCCTCTGACT-3′ (nt 3906–3929 of the cDNA), and SP2, 5′-TGAACAAATACGAGCAGCCTGCATA-3′ (nt 3932–3956); chicken AOH SP1, 5′-GCCCAGATACATACAAGATCCCTG-3′ (nt 3742–3765), and SP2, 5′-CGGATTCGTATGGCCTGTGATGAT-3′ (nt 3975–3998); dog AOH2 SP1, 5′-GGGTGAATCTGGAATGTTCTTGGG-3′ (nt 3812–3835), and SP2, 5′-ATCTGGAATGTTCTTGGGATCCTC-3′ (nt 3818–3841); dog AOH3 SP1, 5′-TGAAGAGCCCAGCAACGCCAGAAT-3′ (nt 4055–4078), and SP2, 5′-CAGCAACGCCAGAATGGAT-

TCGAA-3′ (nt 4064–4087). PCR products were subcloned in pCR2.1 and multiple clones were sequenced.

Phylogenetic Analysis—Multiple sequence alignment was performed using the ClustalW program with default settings (Protein Gap Open Penalty, 10.0; Protein Gap Extension Penalty, 0.2; Protein matrix, Gonnet) (34). The multiple alignment was then used to produce a true phylogenetic tree, in the Phylip type output format, always with the ClustalW program that is based upon the neighbor-joining method of Saitou and Nei (35). The tree was then drawn using the Phylodendron software package. The alignment shown in supplemental Fig. 3 was drawn with Color INteractive Editor for Multiple Alignments (66).

RESULTS

The Complement of Avian MOFEs Consists of XOR and Two Proteins of the Aldehyde Oxidase Type, AOX1 and AOH— Chicken XOR is the only avian MOFE for which primary structural information is available (36). We interrogated the genome of *Gallus gallus* present in GenBank[TM] for the presence of other MOFE genes showing structural similarity with mouse aldehyde oxidases, and we identified two potential genetic loci. Partial reconstruction of the exon structure of the genes permitted the design of specific primers that were used for the cloning of two distinct and incomplete MOFE cDNAs by RT-PCR. We named the cDNA isolated from chicken kidney, AOX1, and that cloned from the Harderian gland, AOH. The missing 5′- and 3′-regions of AOX1 and AOH were isolated by 5′- and 3′-RACE experiments.

Chicken AOX1 shows the highest degree of similarity to mouse AOX1 (64% amino acid identity) followed by murine AOH1 (63%), AOH3 (61%), AOH2 (59%), and XOR (52%). The amino acid identity of AOH and the various members of the murine MOFE is equally high and of the same order of magnitude (61% to AOH2 and AOH3; 60% to AOH1; 58% to AOX1; and 48% to XOR). The deduced amino acid sequences of chicken AOX1 and AOH are easily aligned along their entire length with chicken XOR as well as mouse AOX1 and AOH1 (supplemental Fig. 1).

Chicken AOX1 and AOH are characterized by the typical tripartite structure of all MOFEs, in which three conserved domains of 25, 45, and 85 kDa are observed from the amino to the carboxyl terminus. The structural domains are connected by ill-conserved hinge regions. Chicken AOX1 and AOH are *bona fide* MOFEs, because they show the fingerprint sequence of all the proteins capable of binding MoCo (1). This sequence is located in the large 85-kDa domain of the two proteins that is likely to also accommodate the substrate-binding site.

The 45-kDa domain of chicken XOR contains a conserved amino acid sequence that serves as the binding site for NAD. This sequence is characterized by the presence of a Tyr residue (408 amino acids), which is retained in all the XOR sequences so far determined and is covalently labeled by a NAD analog (37, 38). This Tyr is substituted by a variable amino acid in rodent AOX1, AOH1, AOH2, and AOH3 (25, 27, 28), as well as in all the other aldehyde oxidases of plant and animal origin for which sequence data are available. Neither AOX1 nor AOH of chicken origin shows the presence of a Tyr residue in the

sequence corresponding to the NAD-binding site of XOR, supporting the concept that the two avian proteins are MOFEs of the aldehyde oxidase type. As such, chicken AOX1 and AOH are predicted to be unable to oxidize hypoxanthine and xanthine which are specific XOR substrates. In line with this prediction, the critical glutamic acid and arginine residues responsible for the positioning of hypoxanthine and xanthine into the substrate pocket of bovine XOR (Glu-802 equivalent to Glu-805 and Arg-880 equivalent to Arg-883 in bovine and mouse XOR, respectively) (3) are substituted by different amino acids in chicken AOX1 (Leu-813 and Ile-809) and AOH (Val-891 and Tyr-887). Interestingly, Glu-1261, an amino acid playing a crucial role in the substrate pocket of bovine XOR (3) and conserved in all the known MOFEs, is present also in chicken AOX1 and AOH (Glu-1272 and Glu-1268, respectively).

Finally, chicken AOX1 and AOH demonstrate the presence of conserved regions that fold into the two domains containing the nonidentical 2Fe/2S redox centers typical of all MOFEs. The amino-terminal domains of AOX1 (1–164 amino acids) and AOH (1–162 amino acids) contain the eight conserved cysteine residues responsible for the coordination to the iron atoms of the 2Fe/2S cofactors.

Dogs Express XOR as Well as the AOH2 and AOH3 Orthologous Proteins—*In silico* scanning of the Boxer dog (*Canis familiaris*) genome resulted in the identification of potential genes coding for five types of MOFEs. The exon structure of the dog *XOR* gene was entirely reconstructed and the predicted protein determined. The full-length cDNAs coding for two potential aldehyde oxidases were cloned, using RNA extracted from the nictitating gland and the nasal mucosa, respectively. The cloning strategies used are similar to those described in the case of chicken aldehyde oxidase cDNAs.

As observed in the case of avian AOX1 and AOH, the two novel dog proteins are *bona fide* MOFEs, as indicated by the presence of the two spectroscopically nonidentical 2Fe/2S redox centers, the FAD-binding region and the MoCo-binding/substrate pocket (supplemental Fig. 2). The protein encoded by the cDNA isolated from the dog nictitating gland is 82, 65, 64, 60, and 48% identical to murine AOH2, AOH3, AOH1, AOX1, and XOR, respectively. The dog cDNA cloned from the nasal mucosa codes for a polypeptide that shares the highest level of amino acid identity with mouse AOH3 (84%) followed by AOH1 (65%), AOH2 or AOX1 (63%), and XOR (52%). Based on these results, we named the nictitating gland and the nasal mucosa MOFEs, AOH2 and AOH3, respectively.

In line with the aldehyde oxidase nature of the two proteins, the FAD domains lack the tyrosines responsible for the binding to NAD in XORs. This residue is substituted by a serine in dog AOH2 (Ser-401), similarly to what observed in the rodent orthologous proteins. In contrast, the same tyrosine is substituted by a leucine in dog (Leu-403), mouse, and rat AOH3. As to the two glutamic acids and the arginine residues involved in the oxidation of hypoxanthine and xanthine, Glu-802 is substituted by valine in both dog AOH2 and AOH3 (Val-805 and Val-806), whereas Glu-1261 is conserved in the two canine proteins (Glu-1260 in AOH2 and Glu-1268 in AOH3). This is exactly what is observed in the mouse and rat orthologs. As expected, the crit-
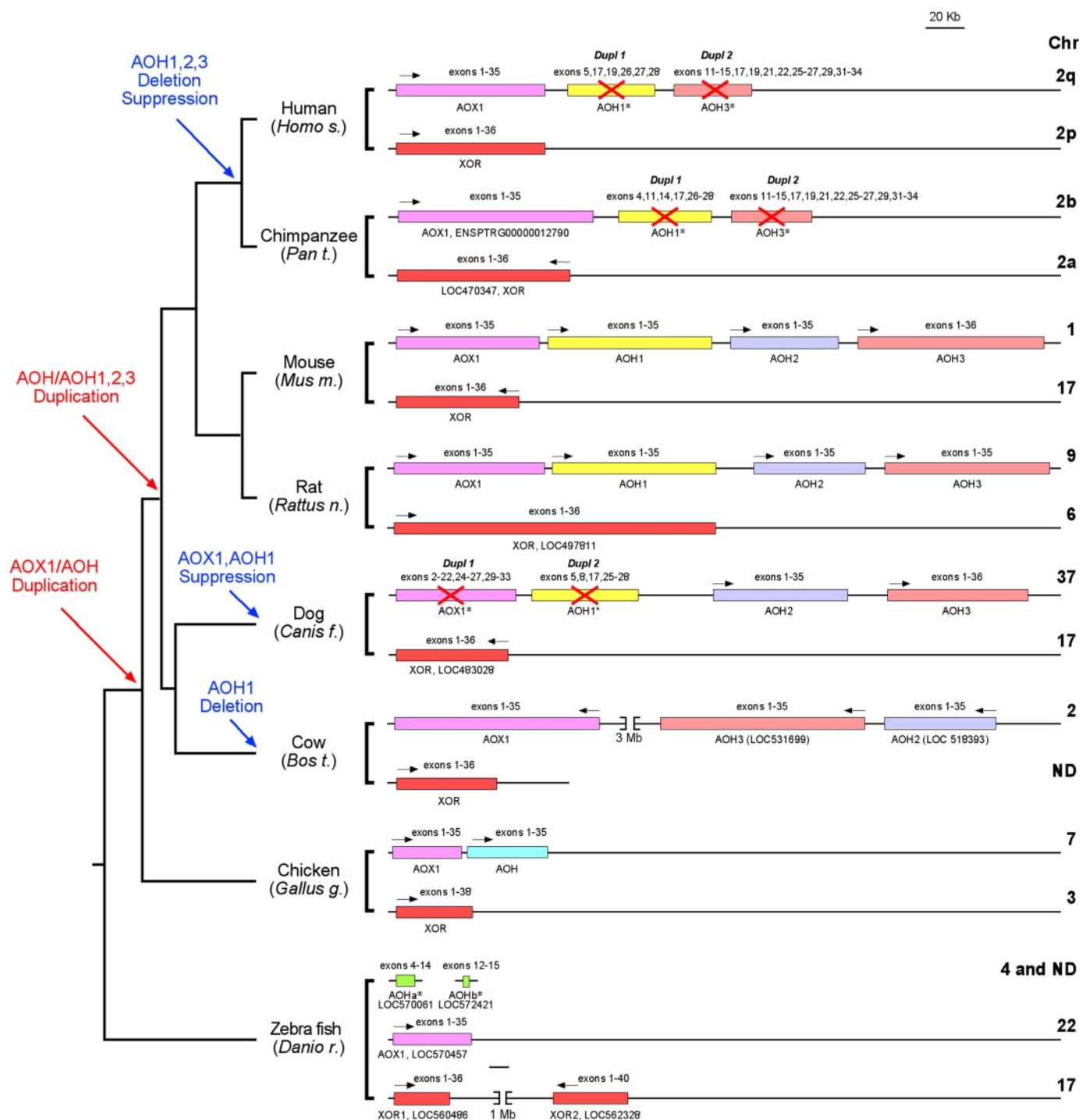
FIGURE 1. **Molybdo-flavoenzyme genes in vertebrates.** The figure shows a schematic representation of the MOFE genes in vertebrates for which complete or almost complete genomic data are available. Orthologous genes are indicated with the same color. The direction of transcription relative to the strand is indicated by *arrows*. Genes transcribed from the upper strand are indicated by an *arrow pointing rightward*, and the *arrows* representing genes transcribed from the lower strand point *leftward*. The number of exons is indicated above each gene. The chromosomal location of the various genes is indicated on the right. *ND* indicates chromosomal position not yet determined. Pseudogenes are crossed in *red* and indicated by an *asterisk* in proximity of the name. Whenever the structure of the gene is predicted solely on the basis of the genomic sequence, and the corresponding cDNAs have not been isolated and sequenced, the GenBank™ locus number (*LOC*) is indicated. The rooted phylogenetic diagram on the left indicates the relative evolutionary distance between the various animal species considered (67).

ical arginine present in the substrate pocket of all the XORs (Arg-883 in the mouse protein) is not conserved in dog AOH2 and AOH3. It is worth noticing that this residue is substituted by a phenylalanine in rodent and dog AOH2 (Phe-886) or AOH3 (Phe-893).

The Chicken Aldehyde Oxidase Cluster Consists of Two Active Genes with Conserved Exon-Intron Junctions Mapping to the Same Chromosome—Computational analysis of the chicken and dog genomes permitted the definition of the chromosomal location and exon structure of the *XOR, AOX1, AOH, AOH2,*

and *AOH3* genes. A schematic representation of the *MOFE* gene complement in chicken and dog is shown in Fig. 1.

The chicken *XOR* gene has the typical 36-exon structure observed in humans, mice, rats, and bovines (4, 23, 39, 40) and maps to chromosome 3. The *AOX1* gene is located on chromosome 7, is at least 40 kbp long, and consists of 35 coding exons. Except for exon 16, whose splice donor site is atypical (GC), all the other exons are interrupted by typical exon-intron junctions conforming to the AG/GT rule for the splicing of nuclear pre-mRNA (supplemental Table 1). The *AOH* locus is also on chromosome 7, has an overall length of at least 42 kbp, is characterized by the same number of coding exons as *AOX1,* and does not show atypical exon-intron junctions (supplemental Table 2). Both genes are transcribed in the same direction. Exon 35 of *AOX1* ends ~4 kbp upstream of the first exon of the *AOH* gene. Notably, the exon/intron structures of the *AOX1* and *AOH* genes determined on the basis of the nucleotide sequence of the two cloned cDNAs are different from those present in GenBank™ and predicted by implementation of appropriate algorithms (LOC424071 and LOC424072). *AOX1* exon 7 is not predicted, whereas exons 21 and 22 are much longer in LOC424071. Other differences are observed in exons 3, 11, 34, and 35. As to *AOH*, exons 2, 12, 13, 24, and 28 are not predicted in LOC424072. In addition, exons 1, 7, 20, and 35 are different. When the sequences of the chicken AOX1 and AOH proteins are compared and the intron/exon junctions of the corresponding genes are aligned, a striking conservation is observed (supplemental Fig. 1). A similar analysis performed with XOR demonstrates conservation of 33 of 35 intron-exon junctions. Conservation is not limited to the position but extends to the type of junctions (type 0, I, or II). No trace of further *MOFE* duplications is evident on chicken chromosome 3.

The Dog Aldehyde Oxidase Cluster Consists of Two Active Genes and Two Pseudogenes—Fig. 1 shows that the dog *XOR* gene maps to chromosome 17, whereas *AOH2* and *AOH3* are located on chromosome 37 at a short distance from each other. Dog *XOR* is a 36-exon gene with a minimal length of 59 kbp. AOH2 consists of 35 exons (supplemental Table 3) for a total length of 60 kbp. *AOH3* is 79 kbp long and composed of 36 exons (supplemental Table 4). The exon-intron junctions of these genes are almost superimposable (supplemental Fig. 2) and show the same positional conservation already observed in the case of chicken. Similar to what was reported for the mouse ortholog (25), the extra exon in *AOH3* contains the last portion of the 3′-untranslated region of the corresponding transcript. Dog *AOH2* and *AOH3* are separated by ~12 kbp and transcribed in the same direction. Implementation of the TBlastn algorithm using the amino acid sequence of mouse *AOX1* and *AOH1* demonstrates the presence of two DNA additional regions of homology (*Dupl 1-AOX1** and *Dupl 2-AOH1**), 34 kbp upstream of the *AOH2* gene. The two regions are separated by ~10 kbp and do not seem to represent genes coding for functional MOFEs.

Dupl 1-AOX1* spans ~66 kbp and consists of 30 recognizable exons, equivalent to exons 2–22, 24–27, and 29–33 of the rodent *AOX1* genes. A complete sequence coding for a typical AOX1 protein cannot be predicted from the exon structure, given the presence of multiple in-frame stop codons in the open

reading frames determined. Nevertheless, some of these exons are transcribed, albeit at low levels, in certain tissues. In fact, RT-PCR experiments on dog liver and kidney poly(A$^+$) RNA, using primers designed against sequences of the putative *Dupl 1-AOX1** exons, resulted in the amplification of cDNA fragments. However, the sequence of these cDNAs could never be extended in the 5′-direction by RACE. In particular, we could never extend this cDNA beyond the 5′ boundary of exon 6. Thus, *Dupl 1-AOX1** does not seem to code for a catalytically active protein. Lack of a translation product is supported by the absence of protein bands after Western blot analysis of dog liver extracts with a polyclonal anti-bovine AOX1 antibody (see Fig. 4).

Dupl 2-AOH1* is ~51 kbp long and contains recognizable exons (5, 8, 17, 25–28) equivalent to the rodent *AOH1* counterparts. The exons of *Dupl 2-AOH1** are not transcribed, as no cDNA fragment could ever be isolated, despite the use of various PCR primers and RNA from different organs and tissues. In conclusion, *Dupl 1-AOX1** and *Dupl 2-AOH1** are likely to be pseudogenes, which represent the vestiges of rodent *AOX1* and *AOH1*.

The Prediction MOFE Genes in Other Vertebrates Indicates Multiple Gene Duplication and Suppression Events—The chromosomal localization and the general organization of the MOFE loci identified in other vertebrates are summarized in Fig. 1. The structure of the *Danio rerio XOR* and aldehyde oxidase genes is based on the exon prediction available in GenBank™. We reconstructed the structure of bovine and simian *MOFE* genes as well as putative protein products (supplemental Fig. 3) by interrogating the corresponding genomes for sequences similar to mouse XOR and aldehyde oxidases, using the TBlastn or the Blastn algorithms. The rodent and human genomes serve as a comparison for the other species.

The marine organism and least evolved vertebrate, *D. rerio*, is characterized by the presence of two distinct *XOR* (*XOR1* and *XOR2*) genes on chromosome 17 and a single aldehyde oxidase gene (*AOX1*) on chromosome 22. Interestingly, even in this fish, all the *MOFE* genes consist of 35 exons whose junctions are largely conserved relative to all the other vertebrates. Short stretches of nucleotide similarities with *XOR* and aldehyde oxidases of various origin are also present on chromosome 4 and an as yet unclassified chromosomal segment. These last two sequences consist of DNA stretches bearing similarities with exons 4–14 and 12–15, respectively, and are unlikely to code for functional MOFE proteins.

Bovines show a peculiar composition and arrangement of the aldehyde oxidase gene cluster. The cluster is composed of three genes with similarity to rodent *AOX1*, *AOH2,* and *AOH3*. *AOX1* is separated from the other two aldehyde oxidase genes by ~3 Mb, which is a long distance relative to what was observed in the cluster of the other vertebrates analyzed. The *AOH2* and *AOH3* orthologs are transcribed on the same strand as *AOX1*. However, the relative position of the three genes in bovines and other mammals is different. The data available indicate the absence of genomic sequences with similarity to rodent *AOH1*.

As reported, the genomes of mice and rats contain four active aldehyde oxidase genes, *AOX1*, *AOH1*, *AOH2* and

AOH3, clustered on chromosome 1 in mice and chromosome 9 in rats (25). The single *XOR* genes map to chromosome 17 in mice and chromosome 6 in rats. Incidentally, reconstruction of the latter gene indicates the presence of a long (114 kb) intron 4 (GenBank™, LOC 497811), which is not observed in any of the other vertebrate species.

The two functional human *XOR* and *AOX1* genes map to chromosome 2q and 2p, respectively (23, 24). At a short distance from *AOX1*, there are two duplicated DNA regions, which we named *Dupl 1* and *Dupl 2* in a previous publication (1), with evident similarity to the aldehyde oxidase genes. *Dupl 1* contains recognizable exons with highest similarity to exons 5, 17, 19, 26, and 27 of mouse *AOH1*. Sequences with highest similarity to exons 11–15, 17, 19, 21, 22, 25–27, 29, and 31–34 of mouse *AOH3* are evident in *Dupl 2*. As originally proposed, *Dupl 1* and *Dupl 2* are likely to be pseudogenes. Now, based on cluster analysis with all the known mammalian aldehyde oxidases (data not shown), we propose that they represent the vestiges of the *AOH1* and *AOH3* genes that underwent a process of genetic suppression. Interestingly, the human genome is devoid of an *AOH2* equivalent.

Not surprisingly, the characteristics of the simian and human *MOFE* genes are almost identical. In fact, *Pan troglodytes XOR* maps to chromosome 2a, whereas chromosome 2b contains *AOX1* and the DNA regions corresponding to human *Dupl 1* and *Dupl 2*.

Phylogenetic Analysis of the Aldehyde Oxidase Proteins Supports Divergent Gene Duplication Events during Evolution—Fig. 2 shows an unrooted phylogenetic tree obtained after comparison of plant and animal XOR as well as aldehyde oxidase proteins (supplemental Fig. 3 for the alignment) for which amino acid sequences are available or can be deduced from the corresponding genomes (see supplemental Table 5 for the list of the protein sequences and accession numbers). The central portion (Fig. 2, *blue*) of the phylogram is dominated by the presence of the characterized or putative XOR enzymes identified in the plant and animal species considered. The XOR branching pattern reflects the phylogenetic relationships of living organisms. Notably, the genomes of *Arabidopsis thaliana* and *D. rerio* contain genes coding for two predicted XOR proteins of extremely high amino acid identity.

Our phylogenetic tree demonstrates two distinct groups of aldehyde oxidases. Indeed plant, insect, and flatworm aldehyde oxidases (Fig. 2, *yellow*) form a distant cluster relative to the vertebrate counterpart (Fig. 2, *green*), consistent with divergent evolution from a common XOR ancestor (1). This confirms and extends the hypothesis of Rodriguez-Trelles *et al.* (41) that aldehyde oxidases evolved twice independently from two different *XOR* paralogs, the second time in chordates, before the diversification of vertebrates.

In the vertebrate aldehyde oxidase cluster, putative AOX1 orthologous proteins are present in all the classes (fishes, amphibians, birds, and mammals). The position of AOX1 in the tree suggests that the corresponding gene is the precursor of all the subsequent duplications. The first evidence of an aldehyde oxidase duplication is present in birds. Interestingly, the product of this duplication (AOH) is more related to mammalian AOH1, AOH2, and AOH3 than to AOX1, suggesting ancestor-

ship. In mammals there is a clear definition of three further aldehyde oxidase isoenzymatic forms: AOH2, AOH1, and AOH3 in order of distance from avian AOH. Overall, the aldehyde oxidase clusters have longer branches than the XOR counterparts, demonstrating faster evolution rates after separation from the ancestor.

Purification of Chicken Kidney AOX1 Demonstrates That the Protein Oxidizes Retinaldehyde to Retinoic Acid—The existence of active aldehyde oxidase enzymes in vertebrates other than mammals has not been proven. To this purpose and as a first step in the characterization of the protein products corresponding to the aldehyde oxidase transcripts identified in chicken and dogs, we focused on avian AOX1. We chose kidney as the primary source of the enzyme, as this organ contains relatively abundant amounts of the AOX1 transcript (see Fig. 3A). Purification of the AOX1 protein was followed by measurement of RAL oxidase activity, as RAL is a natural substrate of the enzyme in other mammalian species (26, 42). Quantitative Western blot analysis was also conducted on all the purified fractions with polyclonal antibody raised against bovine AOX1. The purification scheme is the same used for other types of aldehyde oxidases (25, 27, 28).

In a typical experiment, the calculated RAL oxidase-specific activity of the purified protein was 5.0 units/mg as compared with 0.012 units/mg of the initial cytosolic extract. The overall purification factor was 417-fold. In contrast, the final purification factor calculated by quantitative Western blot analysis was only 48.7. This suggests that the original kidney extract contains large proportions of catalytically inactive enzyme (~98%). The phenomenon may be the result of intrinsic enzyme instability, as observed in at least three independent purification experiments. Alternatively, it may reflect the real presence of a large pool of inactive enzyme in chicken kidney. This is not unprecedented, as high levels of catalytically inactive XOR have been reported in milk (10, 44). In this biological fluid, inactive XOR results presumably from the loss of the terminal MoCo sulfur ligand, which is likely to be present in chicken AOX1, as well as many other mammalian aldehyde oxidases. The overall yield of purified chicken AOX1 is 1 order of magnitude lower than observed in the case of the bovine or mouse counterparts (25, 27, 28). The phenomenon is the consequence of the low affinity of catalytically active chicken AOX1 for the benzamidine-Sepharose chromatography support (data not shown). Nevertheless, the method results in the recovery of a highly purified and catalytically active form of AOX1.

Purified RAL oxidase co-elutes with a single protein band of ~150 kDa, as assessed by electrophoresis under reducing and denaturing conditions. The apparent molecular weight of purified RAL oxidase represents the monomeric subunit of the enzyme, as predicted from the translation product of the AOX1 cDNA. Upon Western blot analysis, the purified RAL oxidase band is recognized by the anti-AOX1 antibody. Polyclonal anti-rat XOR antibodies (13) cross-react with a similar 150-kDa band throughout the procedure until the benzamidine-Sepharose purification step. This indicates that the final AOX1 preparation does not contain contaminations of the structurally and functionally related XOR protein. To establish its identity to chicken AOX1, the purified protein was trypsinized after
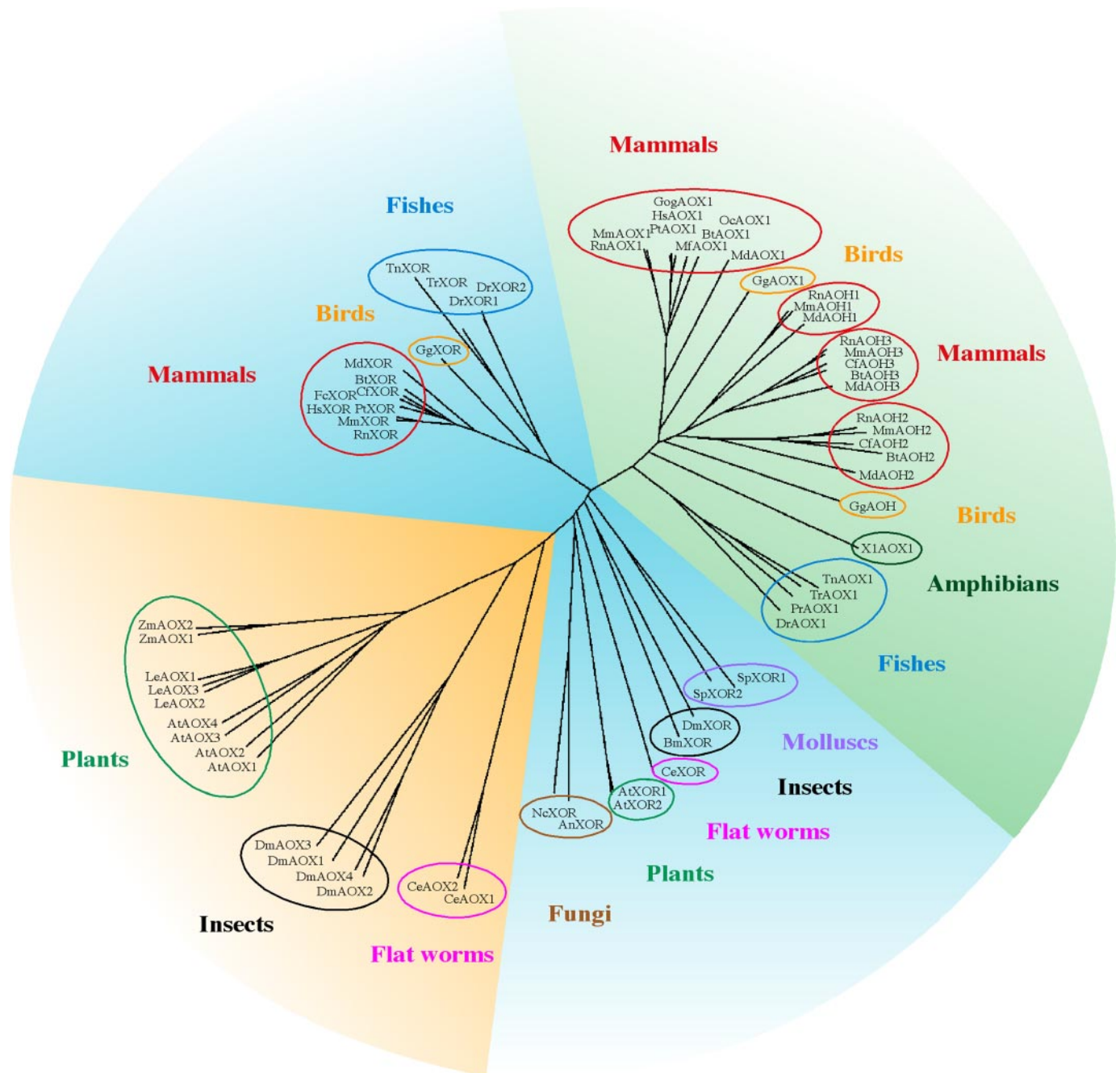
FIGURE 2. **Phylogeny of eukaryotic molybdo-flavoenzymes.** An unrooted dendrogram was obtained by the Phylip method after a ClustalW computer-aided alignment of the indicated proteins. Besides *G. gallus* (*Gg*) AOX1, AOH, and *C. familiaris* (*Cf*) AOH2 and AOH3, the following amino acid sequences were considered: *A. nidulans* (*An*) XOR; *N. crassa* (*Nc*) XOR; *A. thaliana* (*At*) AOX1–4, as well as XOR1 and XOR2; *Lycopersicon esculentum* (*Le*) AOX1, AOX2, and AOX3; *Rattus norvegicus* (*Rn*) AOX1, AOH1, AOH2, AOH3, and XOR; *Mus musculus* (*Mm*) AOX1, AOH1, AOH2, AOH3, and XOR; *P. troglodytes* (*Pt*) XOR; *Homo sapiens* (*Hs*) AOX1 and XOR; *Macaca fascicularis* (*Mf*) AOX1; *Pongo pygmaeus* (*Gog*) AOX1; *Felis catus* (*Fc*) XOR; *Bos taurus* (*Bt*) AOX1, AOH2, AOH3, and XOR; *G. gallus* XOR; *C. familiaris* XOR; *T. rubripes* (*Tr*) AOX1 and XOR; *D. rerio* (*Dr*) AOX1, XOR1, and XOR2; *D. melanogaster* (*Dm*) AOX1–4 and XOR; *Bombyx mori* (*Bm*) XOR; *C. elegans* (*Ce*) AOX1, AOX2, and XOR; *Zea mays* (*Zm*) AOX1 and AOX2; *Oryctolagus cuniculus* (*Oc*) AOX1; *Strongylocentrotus purpuratus* (*Sp*) XOR1 and XOR2; *X. laevis* (*Xl*) AOX1; *Tetraodon nigroviridis* (*Tn*) AOX1 and XOR; and *Monodelphis domestica* (*Md*) AOX1, AOH1, AOH2, AOH3, and XOR.

reduction and carboxymethylation, and the tryptic digest was subjected to MALDI-TOF mass spectrometry. Supplemental Table 6 demonstrates that 54 peptides could be unequivocally identified on the basis of the masses of the tryptic fragments predicted from the open reading frame of the cloned chicken AOX1 cDNA. The other sequences listed are likely to be mixtures of the indicated peptides characterized by identical masses. For all peptides identified, the difference between the calculated and experimental masses is less than 0.05 mass units. Altogether, the identified peptides cover ~55% of the entire sequence of chicken AOX1. A computer-assisted search in the NCBI Protein Database using the masses determined for the 50 most abundant identified peaks did not result in any significant hit. This demonstrates that the protein band with RAL oxidase activity corresponds to chicken AOX1. Most interestingly, the MALDI-TOF analysis did not reveal any tryptic peptide specific
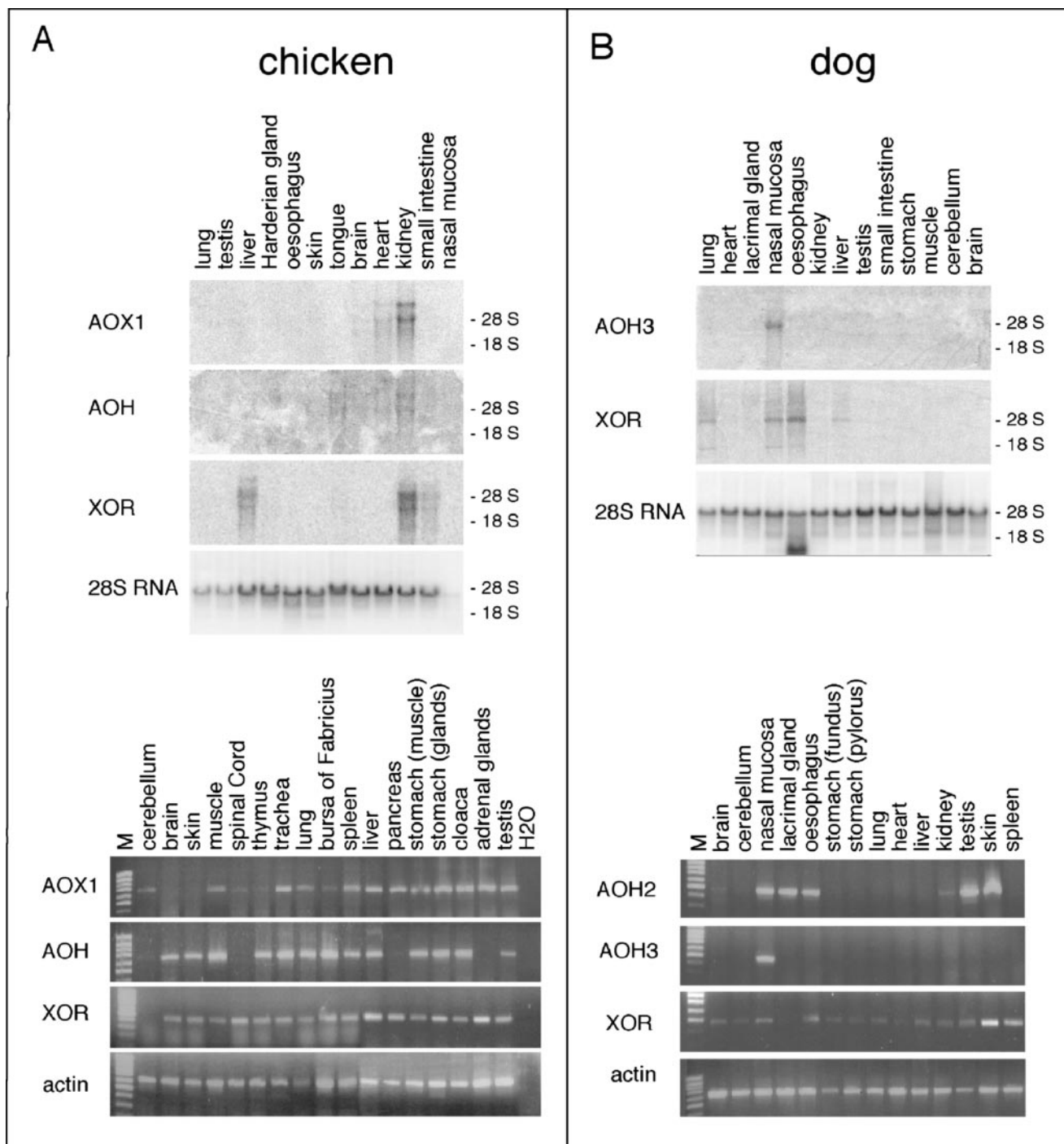
FIGURE 3. **Tissue-specific expression of chicken AOX and AOH and dog AOH3 and XOR.** The indicated tissues were dissected and total RNA extracted. *A, upper panels*, Northern blot analysis of chicken AOX1, AOH, and XOR. Equal amounts (20 μg) of total RNA were run on a 1% agarose gel, blotted on separate nylon filters that were hybridized with radiolabeled chicken AOX1 (nt 3442–4035), AOH (nt 2909–4064), and XOR (nt 3476–3929) cDNA fragments, and oligodeoxynucleotide that recognizes 18 S ribosomal RNA (5′-ACGGTATCTGATCGTCTTGGAACC3′). The autoradiograms of the experiment are shown. The position of the 18 S and 28 S ribosomal RNAs is indicated on the *right*. *Lower panels,* semiquantitative amplification of chicken AOX1, AOH, and XOR cDNAs by RT-PCR. Equal amounts of total RNA (1 μg) were reverse-transcribed and subjected to PCR amplification (30 cycles) using primers specific for the cDNAs encoding: chicken AOX1 (5′-ACGATGCAAATATGGAAT-GGGAGAA-3′, nt 3442–3466, and 5′-CTGTTCAGGTCTCATGCATTCTGG-3′, complementary to nt 4012–4035), AOH (5′-CATGTACAGAGGAGTTAACCGGAC-3′, nt 2909–2932, and 5′-TTCAGAACAGGCAGCTCCAT-3′, complementary to nt 3538–3557), XOR (5′-GGATGGCTGTTCATAATGCATGTC-3′, nt 3476–3499, and 5′-TCTGTTGGGAT-GTCTCCAAATGCT-3′, complementary to nt 3906–3929). Amplification of the chicken β-actin cDNA was used as a positive control of the experiment. The amplified bands were run on a 1% agarose gel and stained with ethidium bromide. *M* indicates DNA molecular weight markers (DNA ladder, 100–1,000 bp); H$_2$O indicates negative controls for the amplification reactions run in the absence of RNA. *B, upper panels*, Northern blot analysis of dog AOH3 and XOR. The experiment was performed exactly as in *A* using dog AOH3 (nt 2916–4116), XOR cDNA (nt 1780–4040) fragments, and oligodeoxynucleotide that recognizes 18 S ribosomal RNA as probes. *Lower panels*, amplification of dog AOH2, AOH3, and XOR cDNAs by RT-PCR. The experiment was performed as in *A* using primers specific for the cDNAs encoding AOH2 (5′-CCCAGTTGAATGATGCCTTACATC-3′, nt 961–984, and 5′-TGGCTTGCAGAGACAACAGTGGAG-3′, complementary to nt 1424–1447), AOH3 (5′-CCCAAGTGAAAGACATTTTGGCTG-3′, nt 1085–1108, and 5′-TTCTGTGCACTGACCATGACAGCC-3′, complementary to nt 1548–1571), and the putative XOR transcript (XM_857591, 5′-TGTGGAAAAGACACTGCATGATGC-3′, nt 963–986, and 5′-CTTGAGAGCTGAGATGGTTCTGTC-3′, complementary to nt 1423–1446). Amplification of the dog β-actin cDNA was used as a positive internal control of the experiment.

for AOH, whose transcript is present in kidney (see Fig. 3*A*). Although the low yields preclude a thorough structural and enzymatic characterization of chicken AOX1, the purified enzyme is capable of oxidizing not only retinaldehyde but also benzaldehyde (data not shown) as the rodent counterpart. Furthermore, chicken AOX1 is devoid of significant xanthine or hypoxanthine oxidase activity.

The Tissue Distribution of the Aldehyde Oxidase Transcripts and Proteins Varies in Different Animal Species—The two richest sources of human and cow AOX1, as well as rodent AOX1 and AOH1, are liver and lung (1). High levels of mouse and rat AOH2 are present in Harderian glands and keratinized epithelia, such as skin and esophagus. Rodent AOH3 is expressed selectively in the nasal mucosa (25). We determined the tissue-specific expression of the chicken and dog aldehyde oxidase genes by Northern blot and RT-PCR analyses. The distribution of the XOR transcripts in the two animal species is included for comparison.

The Northern blot data (Fig. 3*A*, *upper panels*) indicate that chicken AOX1 mRNA is measurable only in kidney and heart. However, the much more sensitive RT-PCR technology (Fig. 3*A*, *lower panels*) highlights the transcript in other organs, including liver. Both the Northern blot and RT-PCR data indicate that the AOH mRNA is co-expressed with AOX1 in several tissues, such as kidney, heart, trachea, liver, and stomach. In addition, brain, skin, and thymus demonstrate selective expression of the AOH mRNA. Northern blot analysis of AOX1 and AOH demonstrates the presence of two specific bands. Although the nature of the more slowly migrating AOX1 and AOH bands is unknown, alternatively spliced forms of mouse aldehyde oxidase transcripts involving the 3′-untranslated region have already been described in mouse (25). Similar to what was observed in rodents (45), the organs expressing the highest levels of chicken XOR mRNA are kidney, liver, and small intestine. Once again and consistent with the rodent situation, RT-PCR demonstrates generalized expression of low levels of this transcript. Compared with the existing literature on other species (5, 6, 8), our data indicate that the chicken and mammalian AOX1 transcripts show different profiles of tissue-specific expression. Thus, although the structural data indicate orthology, chicken and mammalian AOX1 may serve slightly different tissue-specific functions in the two vertebrates. The different tissue distribution of chicken AOH relative to AOH1, AOH2, and AOH3 is consistent with neo-functionalization of the rodent gene duplication products (41).

The expression of dog AOH2 and AOH3 was also studied across a panel of tissues both by Northern blot and RT-PCR (Fig. 3*B*). To avoid cross-hybridization, probes and primers were selected from the sequences with the lowest similarity between the AOH2 and AOH3 cDNAs. The abundance of the dog AOH2 transcript in target tissues is too low to permit detection by Northern blot experiments performed on total RNA. Nevertheless, as revealed by RT-PCR, dog *AOH2* expression is restricted and very similar to what was reported in mice (28). This is consistent with the proposed orthology with rodent *AOH2*. The high levels of AOH2 transcript detected in the lacrimal gland suggest that the corresponding protein plays an organ-specific function in this location. Interestingly, in dogs, this gland is the functional substi-

tute of the rodent Harderian gland. The expression of the canine *AOH3* gene is even more restricted than that of *AOH2*. Indeed, the only tissue where significant amounts of the corresponding transcript are evident is the nasal mucosa. Once again this is in line with the fact that dog and rodent AOH3 are the products of orthologous genes, as large amounts of the protein are synthesized selectively in mouse Bowman glands, the primary exocrine glands present in nasal mucosa. Consistent with what is stated above, although RT-PCR demonstrates the presence of dog AOX1 mRNA fragments, detectable levels of a full-length transcripts were not measured after Northern blot analysis (data not shown). The pattern of *XOR* expression revealed by the same technique is different from that observed in chicken and rodents (45). However, the ubiquitous expression of low levels of XOR mRNA is demonstrated by RT-PCR.

Chicken and Dog Livers Do Not Express Detectable Amounts of AOX1 and Do Not Contain RAL Oxidase Activity—Tissue- or cell-specific expression of a given mRNA is not necessarily associated with synthesis and accumulation of the corresponding protein. Translational and post-translational control of the XOR enzyme was reported in the mouse hepatic tissue and in certain cell lines (13, 45). We verified the presence of the AOX1 and AOH1 proteins in the two chicken tissues, kidney and heart, characterized by the highest levels of AOX1 and AOH mRNAs. Given the importance of liver in the metabolism of xenobiotics mediated by cytosolic aldehyde oxidases (46–49), we extended our analysis of this organ in both chicken and dog. To do this, we used a polyclonal antibody raised against bovine AOX1 that does not recognize XORs of different origin.[6]

First, we determined the cross-reactivity of the anti-bovine antibody with chicken and dog aldehyde oxidases (Fig. 4*A*). Transfection of COS-7 cells, with the cDNAs coding for chicken AOX1 and AOH, dog AOH2 (data not shown) and AOH3, and the mouse AOH1-positive control, demonstrates that the antibody cross-reacts with the four proteins. In contrast, the antibody recognizes neither chicken nor dog XOR (data not shown).

Fig. 4*B* demonstrates that avian heart and kidney contain significant amounts of aldehyde oxidase immunoreactivity. In both cases, this is likely to be the result of AOX1 expression, as suggested by the Northern blot data (Fig. 3*A*). Chicken and dog livers are devoid of aldehyde oxidase immunoreactive bands. In the case of chicken, this indicates that the low levels of AOX1 and AOH transcripts measurable by RT-PCR are insufficient to result in the translation of detectable amounts of the corresponding protein. As to dog, the result is consistent with the lack of detectable amounts of AOX1, AOH2, or AOH3 mRNAs in the hepatic tissue.

That both chicken and dog livers are devoid of aldehyde oxidase immunoreactivity is different from what was observed in humans, mice, and rats, where the hepatic tissue is one of the richest source of AOX1 and/or AOH1 (26, 49, 50). Aldehydes of physiological, toxicological, and pharmacological interest are potential substrates of both aldehyde oxidases of the MOFE family and aldehyde dehydrogenases. The latter family consists of numerous isoenzymes (51) and utilizes NAD^+ as a cofactor for the catalyzed oxidation reaction. To evaluate the functional significance of the aldehyde oxidase deficit in dogs and chicken liver, we determined the ability of cytosolic extracts to oxidize
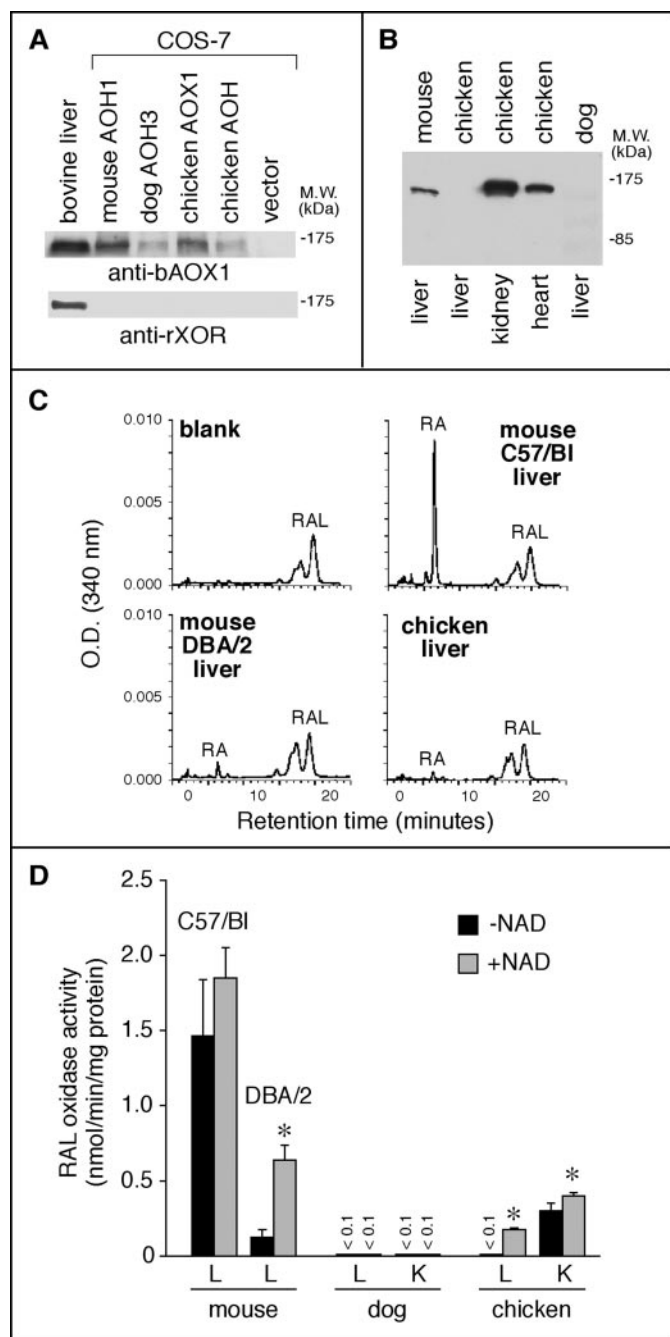
FIGURE 4. **AOX1 and retinaldehyde oxidase activity in chicken, dog, and mouse liver cytosolic fractions.** *A,* cross-reactivity of the anti-bovine AOX1 (*anti-bAOX1*) and anti-rat XOR (*anti-rXOR*) antibodies with the indicated mouse, chicken, and dog proteins. Expression vector (pcMYβ, Invitrogen) containing cDNAs corresponding to the indicated proteins or the void vector (vector) were transfected in COS-7 cells. Two days following transfection, cytosolic extracts were subjected to Western blot analysis with the indicated antibodies. The position of relevant protein molecular mass markers is indicated on the *right. B,* Western blot analysis of aldehyde oxidase immunoreactive proteins in chicken, dog, and mouse tissues. Equal amounts of $100,000 \times g$ cytosolic extracts obtained from the indicated tissues were subjected to Western blot analysis, using anti-bovine AOX1 antibodies. The position of relevant protein molecular mass markers is shown on the *right. C* and *D,* retinaldehyde oxidase activity in mouse, dog, and chicken cytosolic extracts. Equal amounts of desalted $100,000 \times g$ cytosolic extracts obtained from the indicated tissues were incubated for 10 min with retinaldehyde in the absence or presence of exogenously added NAD^+. Samples were extracted in organic solvent and subjected to HPLC analysis. *C,* illustrates representative HPLCs of the indicated samples in the absence of NAD^+: *RA,* retinoic acid. *D* shows the levels of RAL oxidase activity measured using the indicated extracts in the presence (*+NAD*) or absence (*−NAD*) of NAD^+. The

RAL in the presence and absence of NAD^+. For this purpose, liver cytosolic extracts were depleted of NAD^+ by passage on size exclusion columns and subsequently repleted or not with exogenously added dinucleotide. The results were compared with those obtained in cytosolic extracts of C57/Bl and DBA/2 mice that express high and very low levels, respectively, of both AOX1 and AOH1 (26). Fig. 4*C* shows typical HPLCs obtained after incubation of murine, canine, and avian liver extracts with RAL. Fig. 4*D* summarizes the quantitative results obtained with this type of assay. C57/Bl cytosols oxidize RAL to retinoic acid very rapidly. Addition of NAD^+ prior to incubation with the RAL substrate does not result in a significant increase in the amount of retinoic acid produced. The contention is supported by the fact that DBA/2 mice cytosol contains less than 5% of the NAD^+-independent RAL oxidase activity present in the C57/Bl counterpart. This activity is because of the residual AOX1 protein present in the hepatic tissue of this strain (26). A modest increase in the production of retinoic acid is observed upon addition of NAD^+ to the DBA/2 extracts. In the absence of NAD^+, chicken and dog liver extracts do not show detectable RAL metabolizing activity. Addition of NAD^+ causes a slight but significant increase in the production of retinoic acid in chicken liver but not in dog liver. The cytosolic fraction of chicken kidney contains significant levels of NAD^+-independent RAL oxidase activity, which is not further augmented by addition of the dinucleotide. Taken together, our results indicate that the predominant enzymatic activity responsible for the metabolism of RAL in the cytosol of organs such as mouse liver and chicken kidney is an aldehyde oxidase and not a dehydrogenase. In addition, the data confirm the absence of significant amounts of catalytically active MOFEs of the aldehyde oxidase type in the liver of dogs and chicken.

DISCUSSION

In this study, we identify and characterize the cDNAs and the corresponding genes coding for the MOFE synthesized in dog and chicken. Our results demonstrate the presence of one *XOR* and two aldehyde oxidase genes in avians. In contrast, the canine organism is endowed with one *XOR*, two active aldehyde oxidase genes, and an equal number of cognate pseudogenes. Purification of chicken AOX1 permitted the first biochemical characterization of a nonmammalian aldehyde oxidase. Our results are relevant in terms of the biology of aldehyde oxidases. More importantly, they cast new light on the evolution of the *MOFE* family of genes.

MOFEs of the aldehyde oxidase type are believed to represent an important metabolic system capable of oxidizing a large array of endogenous and exogenous substrates (1). The recent demonstration of multiple rodent aldehyde oxidases, characterized by tissue- and cell-specific expression, suggests that specific members of this protein family play a role in the local metabolism of unidentified compounds of physiological, pharmacological, and toxicological significance. The four rodent

results obtained in the absence of NAD^+ are due to AOX1, whereas those obtained in the presence of the cofactor are because of both AOX1 and aldehyde dehydrogenase(s). *, significantly higher than the corresponding value (−*NAD*) ($p < 0.01$ after Student's *t* test).

aldehyde oxidases are structurally related enzymes, have broad substrate specificity, and do not show major differences in terms of substrate preference (25, 27, 28).[6] Among the general substrates of potential physiological relevance, RAL stands out (25, 27, 28, 42) and has been the object of the enzymatic studies reported here. As the rodent counterparts (25, 27, 28, 42), purified chicken AOX1 oxidizes RAL to retinoic acid. RAL is believed to represent the physiological precursor of retinoic acid, a recognized morphogen and an important regulator of the homeostasis of different tissues in the adult. RAL is the oxidation product of vitamin A (retinol) and is thought to be transformed to retinoic acid predominantly by RAL dehydrogenase (52), a specific isoenzyme of the large NAD^+-dependent aldehyde dehydrogenase family (51). In mammals, a major site of RAL metabolism is the liver. Mouse liver cytosol oxidizes RAL to retinoic acid predominantly in a NAD^+-independent manner. Thus, we propose that aldehyde oxidases and not aldehyde dehydrogenases are a major determinant of retinoic acid synthesis in this organ. This metabolic step is catalyzed by an aldehyde oxidase of the AOX1 or AOH1 type, as indicated by the results obtained in C57/Bl and DBA/2 mice (26). Interestingly, the liver of both chicken and dog is devoid of AOX1 or other types of aldehyde oxidase of the MOFE family. As a consequence of this, the cytosol of avian and canine hepatic tissues lack RAL oxidizing activity. At present, we do not know whether the deficit of RAL oxidase observed in chickens and dogs is species-specific or limited to certain strains, as observed in mice (26). Indeed, the data presented in this study were obtained in one strain of chicken (Broiler) and one strain of dogs (Beagle). However, at least in dogs, we favor the former possibility, as the available genomic data demonstrate that the orthologs of the murine *AOX1* and *AOH1* genes are functionally inactive and have been transformed into pseudogenes in both Boxers and Beagles. Regardless of this detail, our data indicate that the metabolism of vitamin A in a major storage organ is different in avians and dogs relative to rodents and humans. The lack of aldehyde oxidase activity in dog liver is of great interest also in a pharmacological and toxicological perspective. In fact, dogs are common experimental models in xenobiotic metabolism studies, and aldehyde oxidases are known to metabolize drugs, like methotrexate (53) and 6-mercaptopurine (54) and environmental pollutants like nitropolycyclic aromatic hydrocarbons.

Definition of the structure of the chicken and dog proteins and corresponding genes contributes to generate a more detailed picture of the evolutionary history of MOFEs. The evolution of *MOFE* genes is complex and characterized by duplication and suppression events, whose traces are present in the genomes of the extant plant and animal species. The amino acid sequence of all MOFEs is highly conserved throughout evolution, although the following four distinct groups of enzymes can be recognized: XORs, aldehyde oxidases, carbon monoxide dehydrogenases, and oxidoreductases of the quinoline and isoquinoline oxidase type (1, 55–58). The last two groups of proteins are present only in bacteria and are not relevant for our discussion, which focuses on the evolution of MOFEs in vertebrates.

XORs and aldehyde oxidases are very similar proteins and are distinguished for their ability or inability to use hypoxanthine and xanthine as substrates (1). From a structural point of view, XORs are identified on the basis of the presence and conservation of two charged amino acids, which are important for the positioning of hypoxanthine and xanthine in the substrate pocket (2, 3). Furthermore, XORs can utilize NAD^+ as a cofactor and act as dehydrogenases (59). As such, XORs are characterized by the presence of a conserved tyrosine in the FAD binding domain (see supplemental Figs. 1 and 2). These structural characteristics were used for the identification of *XORs* and aldehyde oxidases in the genome of various organisms and in the reconstruction of the phylogenetic tree presented in Fig. 2.

The similarity between the amino acid sequences of XORs and the various aldehyde oxidases indicates that the corresponding genes must have evolved from an ancient common precursor (1, 60). This putative precursor enzyme is likely to be more closely related to the extant XORs than to aldehyde oxidases. Indeed, relative to *XORs*, which are already present in bacteria (61, 62), aldehyde oxidases are believed to be a more recent set of genes identified only in multicellular eukaryotic organisms of plant and animal origin (1). However, such a view may be challenged by the recent report describing an aldehyde oxidase from *Methylobacillus* (63).

As suggested previously (1), the first step in the evolution of *MOFEs* must have been the consolidation of the genes responsible for the synthesis of the distinct peptide chains typical of bacterial XOR proteins into a single chain characteristic of eukaryotic XORs. Bacteria, such as *Escherichia coli*, contain structural genes encoding three chains corresponding to the 2Fe/2S-containing amino terminus, the FAD-binding intermediate, and the MoCo/substrate carboxyl-terminal domains of XOR (61). Other bacterial XORs, like *Rhodobacter capsulatus*, consist of $\alpha 2\beta 2$ tetramers (62). Consolidation of *XOR* into a single polypeptide chain must have been an early event during evolution, as fungi like *Aspergillus nidulans* and *Neurospora crassa* are already endowed with a single gene coding for the entire protein subunit (64, 65). This was followed by an increase in the number of the *XOR* exons. The phenomenon is evident when the exon structure of *XOR*s from *A. nidulans* (4 exons), *Drosophila melanogaster* (4 exons), *Caenorhabditis elegans* (16 exons), *D. rerio* (35 exons), and all the other vertebrates (35 exons) is compared. The process of exon multiplication is not progressive and does not follow a simple pattern, as a phylogenetically more ancient organism, like the flatworm *C. elegans,* presents an *XOR* gene with a larger number of exons than the more recent *D. melanogaster* counterpart (1).

Additional and more recent steps in the evolution of *MOFEs* were represented by one or more rounds of duplication resulting in the appearance of up to four distinct genes coding for enzymes of the aldehyde oxidase type. The data presented in this and other papers (41) suggest that the process followed different pathways in plants, insects, and vertebrates. The observation suggests that insect and vertebrate aldehyde oxidase genes are not orthologs, and the corresponding protein products must serve different physiological functions.

The available data on similarity, gene structure, and partial tissue distribution suggest the following evolutionary model for

this group of *MOFEs*. Vertebrate *AOX1* is the proximal paralog of the ancestor *XOR* gene. In fact, the genome of fishes, such as *Takifugu rubripes* and *D. rerio*, contains a single aldehyde oxidase, which is more closely related to rodent, bovine, or dog *AOX1* than to *AOH1*, *AOH2*, or *AOH3* of the same origin, indicating orthology. The transition of sea vertebrates into amphibians, like *Xenopus laevis,* does not seem to be associated with an increase in the number of aldehyde oxidase genes, as this species is characterized by the presence of the sole *AOX1* ortholog. *G. gallus* is the first animal in which the presence of an *AOX1* duplication (*AOH*) is evident. Chicken *AOX1* and *AOH* map to the same chromosome a short distance from each other and represent the first seed of the more complex gene cluster observed in rodents. The presence of an increasing number of aldehyde oxidases from birds to rodents suggests that the process of gene duplication is sequential and proceeds through duplication events involving one gene at a time, not necessarily in the 5′ to 3′ direction of the gene cluster. The *AOH* gene is likely to be the ancestor of mammalian *AOH1*, *AOH2,* and *AOH3.* The phylogenetic analysis presented indicates that the closest relatives to chicken AOH are the AOH2 proteins. Thus it is possible that *AOH1* and *AOH3* are further duplications of the *AOH2* gene. However, the tissue distribution of chicken AOH and mouse AOH2 does not match, indicating different functional roles.

The generation of the two further aldehyde oxidase gene duplications observed in rodents is a relatively recent event and parallels the evolution of terrestrial mammals. The appearance of *AOH1*, *AOH2,* and *AOH3* is likely to be dictated by the need for new tissue- or cell-specific functions. In the case of *AOH2* and *AOH3*, this contention is supported by the very restricted distribution of the two corresponding mouse and rat enzymes in specialized structures like Bowman (25) and Harderian glands. Regardless of the functional significance, the last steps of the process of mammalian *MOFE* gene multiplication preceded the development of placentation, as the genome of opossum (see Fig. 2), a monotreme, is predicted to contain the whole complement of the *AOX1*, *AOH1*, *AOH2,* and *AOH3* orthologs. In this context, it would be interesting to determine the number of aldehyde oxidase genes in marine mammals, such as dolphins and whales.

The process of expansion of the aldehyde oxidase gene cluster stops with the evolution of rodents. Indeed, the canine, primate, and human genomes are characterized by a reduction in the number of the aldehyde oxidases, which is the consequence of selective gene suppression and inactivation events. The traces of these events are recognizable in the genomes of the above-mentioned species under the form of pseudogenes. The aldehyde oxidases inactivated in dogs are *AOX1* and *AOH1*, whereas humans and primates suppress *AOH1*, *AOH2*, and *AOH3*. Humans and primates have lost any evidence of the *AOH2* duplication, although they maintain the vestiges of the rodent *AOH1* and *AOH3* genes. Relative to humans, rodents and dogs are characterized by a much more developed olfactory system, which is of the utmost importance for the survival of these animals in the environment. Given the specific localization in the exocrine glands of the nasal mucosa (25), it is plausible that AOH3 plays a role in the perception of odorants. Indeed, the enzyme might control the duration or the intensity of the olfactory stimuli through rapid metabolism of volatile aldehyde odorants. This function may have become dispensable or redundant in men, leading to the suppression of the *AOH3* gene. This is not unprecedented, because the majority of the genes coding for odorant receptors in mice has undergone a process of progressive inactivation in humans (43). Similarly, suppression of the *AOH2* gene may be related to the disappearance of a functional equivalent of the Harderian gland or to the different characteristics of the skin and oral mucosa in men, primates, and other mammals.

In summary, the aldehyde oxidase gene cluster represents a complex and interesting model of enzymatic evolution. The reasons at the basis of this complexity will become clear as further information on the functional significance of these enzymes is available.

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Avian and Canine Aldehyde Oxidases: NOVEL INSIGHTS INTO THE BIOLOGY AND EVOLUTION OF MOLYBDO-FLAVOENZYMES

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