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Analysis of Aldehyde Oxidase and Xanthine Dehydrogenase/Oxidase as Possible Candidate Genes for Autosomal Recessive Familial Amyotrophic Lateral Sclerosis

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Abstract—*Recently, point mutations in superoxide dismutase 1 (SOD1) have been shown to lead to a subset of autosomal dominantly inherited familial amyotrophic lateral sclerosis (ALS). These findings have led to the hypothesis that defects in oxygen radical metabolism may be involved in the pathogenesis of ALS. Therefore, we decided to analyze other enzymes involved in oxygen radical metabolism for possible involvement in other forms of ALS. We report here analysis of two genes encoding the molybdenum hydroxylases aldehyde oxidase (AO) and xanthine dehydrogenase/oxidase (XDH) for involvement in ALS. Of particular interest, one gene identified as encoding aldehyde oxidase is shown to map to 2q33, a region recently shown to contain a gene responsible for a familial form of ALS with autosomal recessive inheritance (FALS-AR). The AO gene appears to be located within 280,000 bp of simple sequence repeat marker D2S116, which shows no recombination with the FALS-AR locus. The AO gene is highly expressed in glial cells of human spinal cord. In addition, we mapped a gene for XDH to 2p22, a region previously shown to contain a highly homologous but different form of XDH. Neither of these XDH genes appears to be highly expressed in human spinal cord. This evidence suggests that AO may be a candidate gene for FALS-AR.*

INTRODUCTION

Recently, we and others have identified two loci responsible for familial forms of amyotrophic lateral sclerosis (FALS) (1–4). The first of these is inherited in an autosomal dominant fashion and has been shown to be due to mutations in the gene encoding superoxide dismutase 1 (SOD1), located on human chromosome 21 (1, 3). The second locus has been regionally mapped to chromosome region 2q33-2q35 and is inherited in an autosomal recessive pattern (FALS-AR) (2). The gene responsible for FALS-AR has not yet been identified. SOD1 primarily functions as an enzyme that removes superoxide anion and generates hydrogen peroxide. These studies raise the hypothesis that a perturbation in free radical homeostasis can trigger motor neuron degeneration (1, 5, 6). Accordingly, we decided to examine other enzymes that generate superoxide or produce hydrogen peroxide to determine whether they might play a role in familial ALS not associated with mutations in SOD1 or in sporadic ALS. In particular, enzymes that are involved in

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reactive oxygen species metabolism and encoded by genes in the 2q33-q35 chromosomal region are of special interest as possible candidate genes for FALS-AR.

One group of enzymes critical for oxygen radical metabolism is the aldehyde oxidase and xanthine dehydrogenase/oxidase enzymes. Aldehyde oxidase (AO) produces hydrogen peroxide and under certain conditions can catalyze the formation of superoxide (7, 8). It may be important in proper neurotransmitter metabolism (9). Xanthine dehydrogenase (XDH, EC 1.1.1.204) carries out the oxidation of xanthine and hypoxanthine to uric acid, the end product of purine catabolism in humans and some primates. Uric acid may be a quantitatively significant antioxidant in human blood (10). Under certain conditions, XDH is converted into xanthine oxidase (XO) by either oxidation of critical cysteine residues or proteolytic cleavage, each resulting in the removal of the NAD^+ binding domain. In its oxidized form, this enzyme generates significant amounts of superoxide radical molecules (11–13).

The isolation of three cDNAs identified as encoding human XHD/XO has been recently described (14–16). The sequences published by Ichida et al. (14) and Xu et al. (15) are almost identical and have strong homology with mouse (17) and rat (11, 18) XDH genes. Both of these genes have been mapped to human chromosome 2, and the gene studied by Xu et al. (15) has been regionally localized to 2p22 (14, 19). The third human gene, isolated by Wright et al. (16) has homology to the other human forms but differs significantly in some regions (14–16). Moreover, this gene may lack an NAD binding site essential for XDH function. These features suggest that the sequence reported by Wright et al. (16) might be a gene different from XDH. A search of the EMBL DNA sequence data base and comparison of this sequence with 1148 bp of bovine AO reveals that the cDNA isolated by Wright et al. (16) actually shares 94% sequence identity or conservative substitution at the amino acid level. On the DNA level, base pairs 701–1845 of the human cDNA of Wright et al. (16) have over 88.8% sequence homology to base pairs 1–1144 of *B. taurus* AO (EMBL accession number X77527). These data provide strong evidence that the cDNA reported by Wright et al. (16) is the human homologue of AO. AO, like SOD1, is one of a limited number of enzymes that produce hydrogen peroxide.

In this paper we regionally map the AO gene to 2q33. In addition, we physically link this gene to marker D2S116, which has been shown by Hentati et al. (2) to be tightly linked to a recessively inherited form of juvenile onset FALS-AR (2, 20). In situ histohybridization experiments show that AO mRNA is highly expressed in glial cells of the human spinal cord ventral horn. This tissue localization, along with the linkage data and biochemical involvement in the free radical pathway, make AO an excellent candidate gene for FALS-AR. We also regionally map the XDH gene of Ichida et al. (14) to 2p22, the same chromosomal region to which Xu et al. (19) mapped the XDH gene they isolated (15). This rules out both XDH genes as candidates for the form of FALS-AR mapped by Hentati et al. (2).

MATERIALS AND METHODS

Regional Mapping of AO and XDH Genes. Initial chromosomal assignment of the AO and XDH (14) genes was carried out by PCR analysis of a somatic cell hybrid mapping panel as previously described. AO primers are from the 3′ untranslated region, amplifying base pairs 4778–4882 (Genbank Accession L11005). AO1 = 5′-GCTCTTAC-AACTCAATAAAAGGC-3′; AO2 = 5′-CA-AGTCAGAGTAGTTAGGGT-3′. Amplification was done using Amplitaq/Amplitaq buffer (Perkin Elmer). Conditions were 35 cycles of 94°C for 30 sec; 55°C for 30 sec; and 72°C for 30 sec. PCR products were sepa-

rated on 2% agarose gels and stained with ethidium bromide. YACs were isolated using the same primers by previously published methods (21, 22).

FISH was performed as previously published (14) with some modifications. YAC probes were labeled with biotin or digoxygenin. Human Cot-1 DNA was added to block repeat sequences. In some experiments, biotin-labeled alpha-satellite probe detecting the centromere of chromosome 2 was added to the YAC probe mixture (Oncor D2Z). Probe mixtures were preannealed for 1 h at 37°C, placed on predenatured chromosome preparations, cover-slipped, and hybridized overnight at 37°C. Posthybridization washes were done at 43°C in 50% formamide/1× SSC for 15 min and 1× SSC for 8 min. Probe signals were detected with either anti-digoxygenin-FITC (Vector) or avidin-Texas red (Oncor, Inc.) with one amplification as per the manufacturer's directions. Chromosomes were counterstained with DAPI or propidium iodide.

Analysis of YACs for AO and STS Content. STS and DNA marker content of YACs was carried out by PCR. For AO, the AO1 and AO2 primer sets were used. For D2S116, primer sets were D2S116#1 = 5′-CTCCACAAGTTGCTCATAATCC-3′; DsS116#2 = 5′-ACTCTTGCTTCTTCTAGGGA-3′. PCR conditions and gel conditions were as described above.

Pulsed Field Gel Electrophoresis. High-molecular-weight DNA from normal human male lymphoblasts suspended in agarose plugs was digested with either MluI, NotI, or NruI obtained from New England Biolabs. All plugs were dialyzed for 1 h in manufacturer-provided 1× buffer and then digested 14 h at 37°C with 20 units enzyme/μg DNA. Digested plugs were electrophoresed on a Beckman Geneline TAFE system using an 0.75% agarose gel in 0.25 × TBE buffer. The gel was run at 250 V starting voltage with constant current using a 60-sec pulse time for 18 h. DNA was transferred to GeneScreen Plus membrane (Dupont-NEN). PCR products were used as probes by incorporation of [α-^{32}P]dCTP (Amersham, Inc.) with a Random Primed Labeling Kit (Boehringer-Mannheim).

In Situ Histohybridization. Thoracic segments of human spinal cords obtained approximately 2 h postmortem were frozen and stored at −80°C until they were processed. Twelve-micron sections were cut in a cryostat at −20°C and thaw mounted onto silanized glass slides. Oligonucleotides complementary to the AO mRNA molecule were labeled and used for in situ hybridization histochemistry as described earlier (6). Following the hybridization and wash steps, the slides were either placed on a Fuji BAS-III imaging plate for four days and the plate scanned in a Fuji BAS 2000 phosphorimager or coated with NTB3 emulsion and developed two weeks later. Oligonucleotides complementary to the AO mRNA were labeled and used for in situ hybridization histochemistry as described earlier (6). Sense strand probes were used as controls. Oligonucleotide used for in situ histohybridization were:

AO
5′-CTTCTGGAGAAAACAGAGTGCCTC-TTCCCAGATGGCAATCTGTCCTATCT-CTGTG CTGGA-3′
50% AT–50% GC
Dehydrogenase—Ichida et al. (14).
5′-GAGTCCTCAGCAGAGTCTTCTTGT-GCTGCCTTTGGGCTTCCATGGAGCA-GGAGGAACATA-3′
51.6% GC–48.4% AT
Dehydrogenase—Xu et al. (15)
5′-AGTGTTTTGGGCTAAGGTGGGGC-CTCCATGGAGCCAGAAGTGCATATT-GTAGTATCCAGA-3′
50% GC—50% AT

RESULTS

Assignment of AO Gene to Human Chromosome 2. Our initial strategy to map

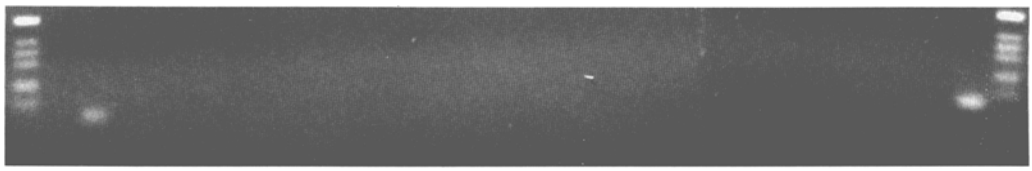

Fig. 1. Somatic cell hybrid mapping panel mapping of the AO gene. A single chromosome mapping panel was used to analyze the chromosomal location of certain STS. This panel consists of 24 cell lines, each typically containing a single human chromosome in a hamster or mouse background. The cell lines comprising this panel have previously been described in Pahl et al. (21). Chromosomes 2–12, 14–19, 21, 22, X, and Y are contained singly in hybrids. Line GMO72994 contains chromosomes 1 and X; R370-22A contains chromosomes 13 and 9; and GM11441 contains chromosomes 20 and a del(5)(15.1p15.3).

the AO gene by PCR utilized a monochromosomal human/rodent somatic cell hybrid panel (21). Primers (AO1 and AO2) were designed to amplify a region from the untranslated 3′ end of the cDNA. Only the hybrid containing human chromosome 2 shows a positive result (Fig. 1).

Regional Mapping of AO Gene. To regionally map the AO gene, we isolated two separate yeast artificial chromosomes (YACs), B136E9 and A188E12, from the library of Burke et al. (23). We then performed fluorescence in situ hybridization (FISH) to human metaphase chromosomes using total DNA from these YACs as probes according to previously published procedures (22). Using chromosomes obtained from normal male lymphocytes, the results of this analysis using the apparently nonchimeric YAC B136E9, shown in Fig. 2a, position the AO gene at 2q33. In this figure, we identified chromosome 2 by coprobing with a chromosome 2-specific centromere probe. Band position was determined by the fractional length method, as previously described (22).

Regional Mapping of XDH Gene of Ichida et al. (14). We used PCR primers

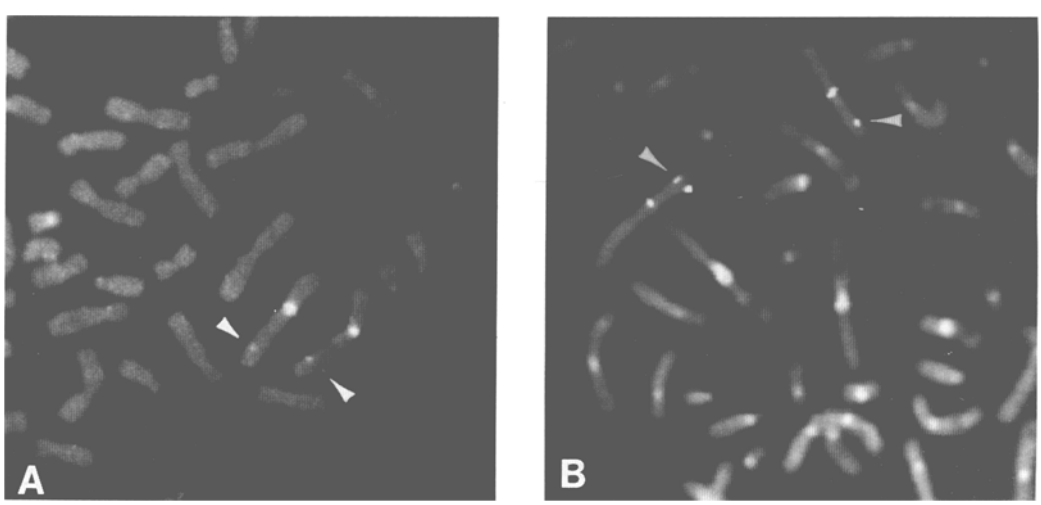

Fig. 2. FISH mapping of the AO (A) and XDH (B) loci to chromosome regions 2q33 and 2p22, respectively. FISH was performed as previously published (22) with some modifications. YAC probes were labeled with biotin or digoxygenin. Human Cot-1 DNA was added to block repeat sequences. Biotin-labeled alpha-satellite probe detecting the centromere of chromosome 2 was added to the YAC probe mixture (Oncor D2Z). Chromosomes were counterstained with DAPI or propidium iodide.

specific for the 3′ untranslated region of the cDNA of the gene mapped to chromosome 2 by Ichida et al. (14) to confirm that this gene indeed maps to chromosome 2 using our somatic cell hybrid mapping panel (data not shown) and to isolate a YAC, B196G6 (22) containing this gene. We used this YAC as a FISH probe on human metaphase chromosomes. As can be seen clearly in Fig. 2b, this gene maps to chromosome region 2p22. Interestingly, this is the same chromosomal region to which Xu et al. (19) have mapped the XDH gene they isolated. Therefore, if these two groups have indeed isolated cDNAs for two different human XDH genes, they both map to the same small region of chromosome 2. Since these genes map to 2p22, neither of them can be considered candidate genes for the FALS-AR locus at 2q33.

Physical Linkage of AO Gene to DNA Marker Showing No Recombination with FALS-AR. FALS-AR in a highly inbred Tunisian population has recently been mapped to a region of approximately 10 cM on chromosome 2 by genetic linkage strategies using a number of highly polymorphic dinucleotide repeat markers from chromosome 2q (2). Three of these markers, D2S116, D2S72, and D2S155, show no recombination with the FALS-AR locus and have individual LOD scores of over 4.5, with a combined LOD score of over 6.0. We tested the AO YAC A188E12 and four other YACs positive for the marker D2S116 (747A1, 800G4, 828E8, and 849B12) from the CEPH MegaYAC library (24) for the presence of AO, D2S116, D2S72, and D2S155 using PCR. As can be seen in Fig. 3, D2S116 is present in the AO YAC A188E12. The size of this YAC, which contains both the 5′ and 3′ ends of the published cDNA sequence (data not shown) is 340 kb, giving this as a maximum distance between D2S116 and the AO gene. In addition, the AO primers amplified DNA from three of the four D2S116 containing MegaYACs. Primer sets for D2S72 and D2S155 failed to amplify DNA from any of these YACs (data not shown).

Physical linkage of AO to D2S116 was confirmed by pulsed field gel electrophoresis (PFGE). Normal human male DNA isolated in agarose plugs was digested with three different rare cutting restriction enzymes, MluI, NotI, and NruI, and separated by PFGE. AO, D2S72, DS116, and D2S155 were used as separate probes for Southern blot hybridization analysis of this DNA. The results of this analysis, shown in Fig. 4, show that AO and D2S116 share DNA bands of the same size with all three enzymes, the smallest being 280 kb. D2S72 and D2S155 appear not to be physically linked to AO/D2S116 or to each other.

Expression of AO Gene in Human Spinal Cord. If mutations in the AO gene are responsible for FALS-AR, the gene should be expressed in ventral horn of human spinal cord. Therefore, we designed oligonucleotide probes from the 3′ untranslated region of the cDNA of the AO gene and performed in situ histohybridization on normal human spinal cord (6). The results (Fig. 5a) show that AO mRNA is indeed expressed in the ventral horn of spinal cord. High-resolution in situ histohybridization (Fig. 5b) shows that by far the most significant expression of the AO gene is in the glial cells, not large motor neurons. Numerous positive cells are also observed in the white matter of the posterior funiculus of the spinal cord (Fig. 5C). We also carried out in situ histohybridization using identical procedures with oligonucleotide probes that are specific for the cDNAs reported by Ichida et al. (14) and Xu et al. (15). We detected some expression of the former in ventral horn and were unable to detect expression of the latter using this approach (data not shown). These results support the hypothesis that the AO gene and the two reported forms of XDH are indeed independent genes with different patterns of expression.

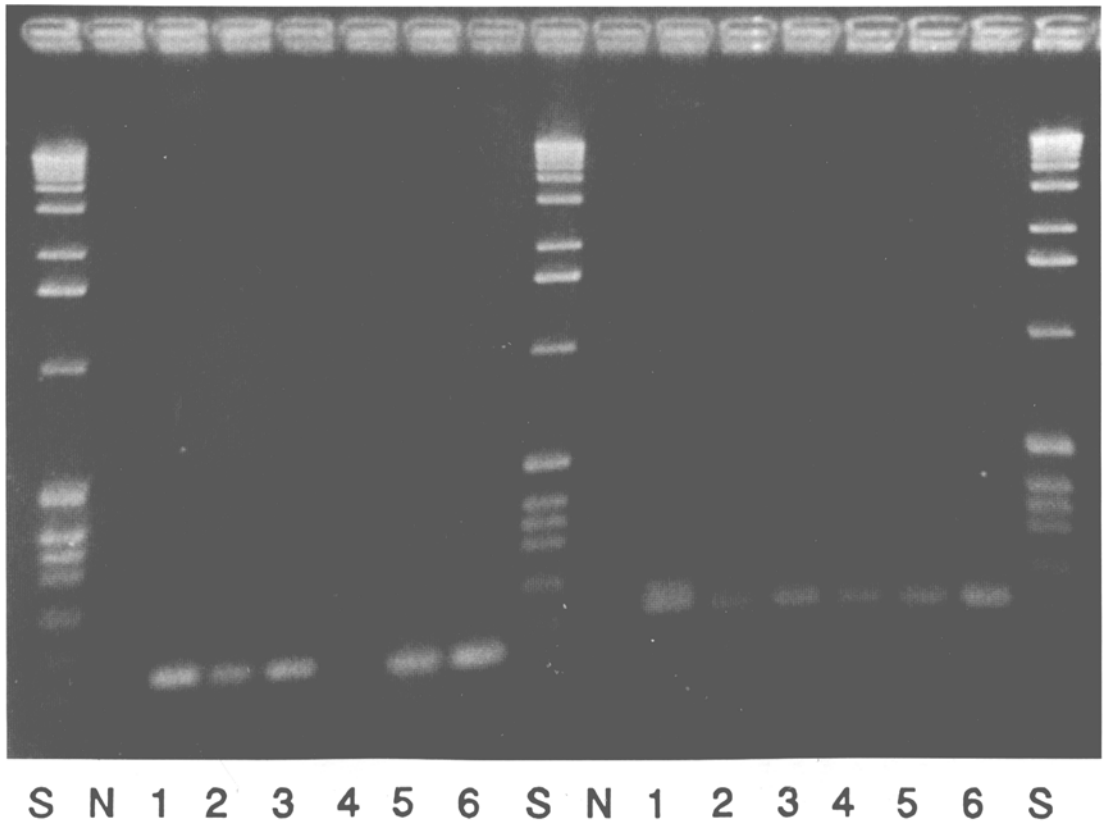

Fig. 3. Analysis of YACs for AO and D2S116 content. PCR amplification from common YACs using primer sets from AO and D2S116. PCR templates for both gels are as follows: N = no template control; 1 = total human genomic DNA, 2 = YAC A188E12; 3 = YAC 747A1; 4 = YAC 800G4; 5 = YAC 828E8; 6 = YAC 849B12; S = BRL 1-kb ladder. Left: gel reactions were amplified using primers AO1 and AO2; right: gel reactions were amplified using primers D2S116#1 and D2S116#2.

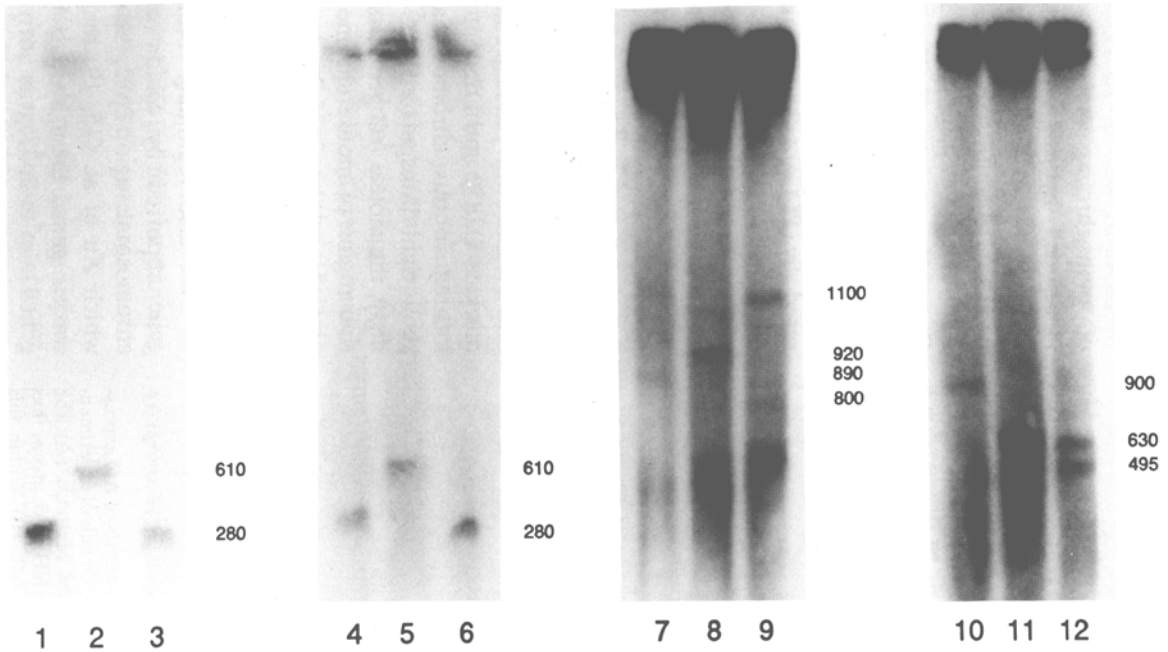

Fig. 4. Pulsed-field gel analysis of AO and chromosome 2 markers. Pulsed-field gel analysis using probes AO (lanes 1–3), D2S116 (lanes 4–6), D2S72 (lanes 7–9), and D2S155 (lanes 10–12) on total human DNA digested with MluI (lanes 1, 4, 7), NruI (lanes 2, 5, 8), and NotI (lanes 3, 6, 9, 12) was carried out as described in Materials and Methods. Numbers to the right of each gel are approximate sizes in kilobases as determined using yeast chromosomal DNA loaded in adjacent lanes.

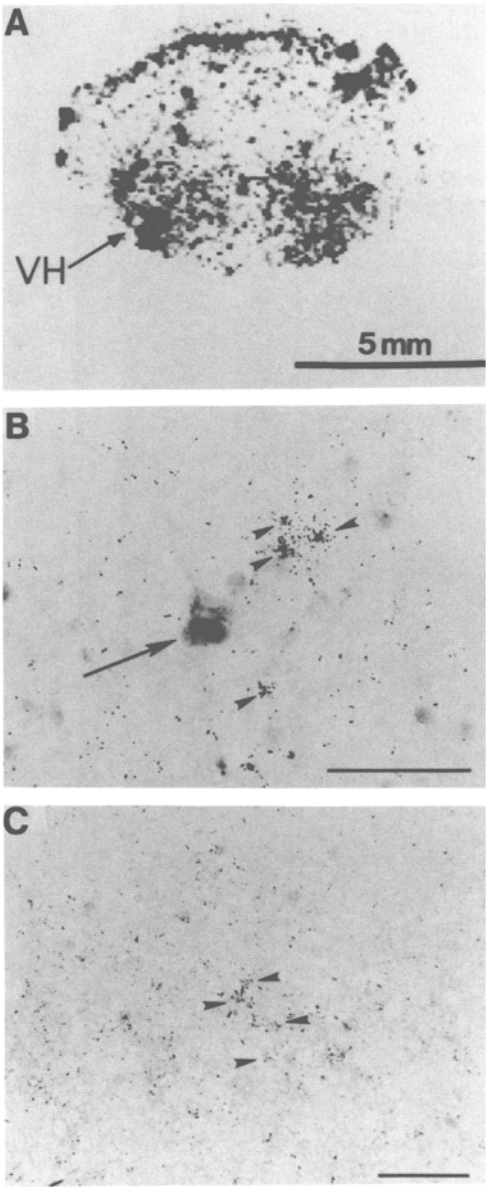

Fig. 5. In situ histohybridization analysis of AO expression in human spinal cord sections. Details of experimental procedures are in Materials and Methods. (A) The ventral horn, indicated by the VH and arrow, has relatively intense expression of AO. (B) Within this structure large motor neurons (large arrow) did not seem to be labeled, but many small cells (arrowheads) were. Numerous positive small cells were also observed in the white matter of the posterior funiculus of the spinal cord (C, arrowheads). Bars in B and C are equal to 50 μm.

DISCUSSION

In this paper, we present data mapping a gene encoding human aldehyde oxidase to chromosome region 2q33 using 3 independent approaches. The initial chromosome assignment was done by PCR using a panel of monochromosomal human–rodent somatic cell hybrids. Confirmation of this assignment and regional mapping was done by FISH using two independent YACs containing at least part of the AO gene. Regional mapping to 2q33 was confirmed by physical linkage to the marker D2S116 by two methods: hybridization to equal-sized high-molecular-weight DNA fragments separated by PFGE after digestion by three different restriction enzymes, and by isolation of four independent YACs from two different libraries that contain both AO and D2S116. The maximum distance between D2S116 and AO appears by this analysis to be 280 kb. This short distance between markers indicates that AO also has tight linkage to the FALS-AR locus. We do not find evidence for physical linkage of AO to markers D2S72 and D2S155, which are also located in this chromosomal region, and show tight linkage to FALS-AR. Interestingly, the mouse AO gene has been mapped to a region of mouse chromosome 1 that is syntenic to the 2q33-35 region of human chromosome 2, confirming the identification of the cDNA reported by Wright et al. (16) as the human cDNA for AO (25, 26).

We also regionally localize the XDH gene reported by Ichida et al. (14) to chromosome region 2p22, the same region to which Xu et al. (15, 19) mapped a highly similar gene. Even though both of these genes map to 2p22, it is not yet known how close they are to each other on the DNA.

Interestingly, AO appears to be expressed in ventral horn glial cells, whereas expression was not detected in large motor neurons, the cells that degenerate in ALS.

Glial cells form some 90% of the cells within the nervous system (27). They traditionally have been understood to support the environment around nerve cells, for example, by regulating the concentrations of free ions in the extracellular spaces of the spinal cord, transporting materials to and from blood vessels, and providing metabolic support for the neuronal membrane (28, 29). More recently, glial cells have been found to be involved in wound repair in the spinal cord (30), to secrete growth factors necessary for the survival of large motor neurons (31), and to remove and inactivate neurotransmitter molecules (32). Some evidence suggests that subtle metabolic changes in glial cells may affect the functions of neurons (32).

It is interesting to speculate on the mechanism by which mutations in AO might lead to motor neuron degeneration and FALS-AR. AO catalyzes the oxidation of aldehyde moieties to acid moieties (9). An important action of AO within the nervous system is its involvement in the catabolic pathway of the catacholamines epinephrine and norepinephrine. Both these neurotransmitters are common in the grey matter of the spinal cord (9, 33), and they depolarize the large motor neurons of the ventral horn (34, 35). Monoamine oxidase acts upon both neurotransmitters to produce dihydromendelaldehyde. AO in turn acts upon the aldehyde to produce dihydromandelic acid (9). Monoamine oxidase is also present in nerve cells and glial cells. Thus, mutations in AO may interfere with the proper metabolism of neurotransmitters essential for the modulation and long-term survival of large motor neurons.

Alternatively, clearly AO is also important for reactive oxygen species (ROS) metabolism, since it produces hydrogen peroxide and, under certain conditions, has been found to catalyze the formation of superoxide radicals (7, 8). Mutations in SOD1, which also produces hydrogen peroxide, lead to ALS. Initial speculation was that mutations in SOD1 might cause ALS due to a decrease in SOD1 activity and hence a possible increase in superoxide and a decrease in hydrogen peroxide production (36). Recent results, including those from studies of transgenic mice, indicate that a simple loss of SOD1 activity is unlikely to be responsible for ALS (5, 37, 38). Evidence has been presented demonstrating that SOD1 mutations associated with ALS enhance apoptosis (39), a finding consistent with the clear involvement of ROS metabolism in ALS (40). These studies also support the hypothesis that it is not a simple loss of SOD1 activity that is responsible for ALS. Similarly, it is hard to see how loss of AO activity and a presumed resultant loss of hydrogen peroxide production in glia would lead to loss of motor neurons. We speculate that alterations in glial cell metabolism of ROS metabolism or neurotransmitters might affect neuronal survival. These observations suggest that AO should be examined carefully as a possible candidate gene for FALS-AR. Such studies are underway.

If mutations in AO are found to lead to ALS, this would strongly reinforce the importance of analyzing other enzymes involved in metabolism of ROS for involvement in ALS and in other neurodegenerative disorders like Parkinson's disease and Alzheimer's disease. Additionally, it would support the hypothesis of environmental influences on ROS metabolism as possible causes of ALS.

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