A Second Superoxide Dismutase Gene in the Medfly, *Ceratitis capitata*

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ABSTRACT

We report the first case of two Cu/Zn *Sod* genes (*ccSod1* and *ccSod2*) that have been cloned and sequenced from an insect, the medfly, *Ceratitis capitata*. Biochemical evidence suggested the presence of two *Sod* genes in the medfly. The two genes are isolated using different molecular strategies: *ccSod1* via cross-hybridization to a genomic library using a heterologous probe and *ccSod2* from cDNA using a homologous probe generated by PCR. Sequence analysis shows that *ccSod1* and *ccSod2* are different genes. The inferred amino sequences show that all essential residues of the active site are strictly conserved, which suggests both genes encode functional Cu/Zn superoxide dismutase (SOD). Phylogenetic analysis by the maximum parsimony method with bootstrap resampling of previously known Cu/Zn SOD reveals two monophyletic groups, vertebrates and insects. The position of ccSOD2 in this phylogeny is undefined with respect to dipteran ccSOD1, vertebrate, plant, fungal, and extracellular Cu/Zn SOD, which suggests that the duplication detected in Ceratitis is ancient, perhaps as old as the origins of the arthropod phylum in the Cambrian more than 500 million years ago. *In situ* hybridization to polytene chromosomes places the genes on different chromosomes, which is consistent with an ancient gene duplication.

THE medfly, *Ceratitis capitata,* is an important agricultural pest for which molecular (ZACHAROPOULOU *et al.* 1992) and genetic maps (MALACRIDA *et al.* 1988) are being constructed. A large number of biochemical loci have been mapped in the medfly (MILANI *et al.* 1989). Oligonucleotide primers have been designed, based on sequence information from other organisms, to amplify by PCR homologous sequences from the medfly. These PCR products are then used as probes for isolating the genes from genomic and cDNA libraries (SCOTT *et al.* 1993). The cloned genes have in turn been located on polytene chromosomes by *in situ* hybridization, thus correlating the genetic with the cytological map.

One gene included in this genome mapping encodes superoxide dismutase (SOD; EC1.15.1.1), which catalyzes the conversion of 0_2^- to $H_20_2 + 0_2$, thus protecting the cell from oxidative damage. In most nonplant eukaryotes, SOD is present in two forms: a mitochondrial enzyme containing Mn and a cytoplasmic one containing Cu/Zn (FRIDOVICH 1986). We cloned a Cu/Zn *Sod* gene, hereafter called *ccSod1,* from a genomic library by cross-hybridization to a *Drosophila melanogaster* probe (KWIATOWSKI *et al.* 1992). Independently, a second *Sod* gene fragment was isolated by PCR from adult medfly cDNA. This fragment was in turn used as a probe to identify a number of clones from a third-instar larval cDNA library. All these clones contained the identical *Sod* sequence, and this gene is herein designated *ccSod2* (GenBank accession L35494).

Earlier biochemical analysis suggested the presence of two Cu/Zn SOD genes in the medfly (FERNANDEZ-SOUSA and MICHELSON 1976). Comparison of the coding sequences, as well as their remote location on the polytene chromosomes, shows that *ccSod1* and *ccSod2* are two different *Sod* genes. We present a comparative analysis of the two *Sod* coding regions, their cytological location, and discuss their phylogeny.

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MATERIALS AND METHODS

General methods: Medfly genomic DNA was isolated from the Benakeion strain by the method of HOLMES and BONNER (1973). Total RNA was prepared by the procedure described by ASHBURNER (1989). cDNA was synthesized following the protocol of Strategene. S. BROGNA provided a third-instar larval Lambda ZapII-cDNA library, which was screened by standard procedures (MANIATIS *et al.* 1982) using nitrocellulose membranes. The prehybridization and hybridization buffers comprised 50% formamide, 6× SSC, 0.1% SDS and 5× Denhardt's; the filters were incubated at 42° for both steps. Two sequential cold washes (5 and 15 min) at room temperature and two hot washes at 65° were carried out in 2× SSC and 0.1% SDS. DNA was sequenced via the chain termination method (SANGER *et al.* 1977).

PCR primers and amplification: Four degenerate primers (I and II, 5′-3′; III a and b, 3′-5′) were synthesized: primer I, 5′-CA-(CT)GG(ACGT)TT(CT)CA(CT)GT(ACGT)CA-3′; primer II, 5′-GG(ACGT)CC(ACGT)CA(CT)TT(CT)AA(CT)CC-3′; primer

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ttgccgctgctggtgtcgatcgtagggcgggccgggtcgcgtgtatatatatgtcatcgc   60
taacatagccagcttagacacacgaatcagtgactaatcgtagtgtaaaagtaatgccaa  120
tataaatgcattacatatgccttaatagtcacattggccttgtgtacggtcagcgccctg  180
gcagagaaaagtgctacaatggaggcaattgcctacgtggagggtcccgtagtgaagggt  240
                  m  e  a  i  a  y  v  e  g  p  v  v  k  g
aatgttacattcatacaaaatggctgctccgagaacgttcacgtgcatgtctatttgact  300
 n  v  t  f  i  q  n  g  c  s  e  n  v  h  v  h  v  y  l  t
ggcttgacaccgggcaaacacggtttccatgtgcatgagaaaggcgatttgacgaatgca  360
 g  l  t  p  g  k  h  g  f  h  v  h  e  k  g  d  l  t  n  a
tgcgccagcaccggcggacacttcaatccggataagatggatcacggggcacctggtgat  420
 c  a  s  t  g  g  h  f  n  p  d  k  m  d  h  g  a  p  g  d
gaagtacgtcacgtaggcgatctcggtaatatagaggcagatgcgaatggcgtcgtcgac  480
 e  v  r  h  v  g  d  l  g  n  i  e  a  d  a  n  g  v  v  d
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 t  t  f  t  d  h  l  i  s  l  t  g  k  r  t  i  v  g  r  g
ttggtggtgcacgaattgaccgatgatttgggcaagggatgccatcccgattcgaagaag  600
 l  v  v  h  e  l  t  d  d  l  g  k  g  c  h  p  d  s  k  k
actggcaatgcaggcggacgtttggcttgcggtgtcatcggtgtcaagtaattaagaaaa  660
 t  g  n  a  g  g  r  l  a  c  g  v  i  g  v  k  *
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aactggaatcaatataaaaatttttgtctttaatcaaatctgcaattaaaaaaaaaaaaaa 1080
aaaaaaaaaaaa                                                  1093
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FIGURE 1.—Nucleotide sequence of the cDNA for the *ccSod2* gene in the medfly, *Ceratitis capitata*. The inferred amino acid sequence is shown below the corresponding codons. The terminating codon is indicated with an asterisk; putative regulatory sequences are underlined; regions corresponding to the PCR primers are shaded. The sequence has been submitted to GenBank/EMBL under the accession number L35494.

IIIa, 5′-CC(AGT)AT(ACGT)AC(ACGT)CC(AG)CA(ACGT)-CC-3′; and primer IIIb, 5′-CC(AGT)AT(ACGT)AC(ACGT)C-C(AG)CA(ACGT)GC-3′. The primers are based on three conserved regions in known SOD enzymes (see Figure 3): primer I from aa44 to aa49 (HGFHVH), primer II from aa62 to aa67 (GPHFNP), and primers IIIa and b cover the region aa147 to aa152 (GCGVIG and ACGVIG, respectively). The reason for the single amino acid difference between primers IIIa and IIIb is that nondipteran SOD enzymes have alanine at position aa147, whereas two known species of Drosophila have glycine. (When the primers were designed, neither the medfly *ccSod1* nor the *Chymomyza amoena* sequences were available.)

Reactions (100 μl) were carried out with 50 pmol of each primer and 1 μg adult medfly cDNA in 7.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 units Taq polymerase (Perkin Elmer-Cetus) in manufacturer's buffer. Samples were overlaid with mineral oil, denatured at 94° for 5 min and then put through 35 cycles of 94°C × 1 min, 53°C × 1 min, 72°C × 1.5 min, followed by a final extension at 72° for 7 min. All PCR reactions were performed with a Perkin Elmer-Cetus thermal cycler.

***In situ* hybridization:** Salivary gland polytene chromosomes were prepared from third-instar larvae for *in situ* hybridization. We followed the methods of ZACHAROPOULOU *et al.* (1992), except that we used the avidin complex (ABC Elite Kit, Vectostain) instead of horseradish peroxidase.

RESULTS

PCR, library screening and genomic Southern analysis: Neither primer I nor II in combination with IIIa resulted in any PCR amplification product from the adult medfly cDNA template. Amplification with primers I and IIIb resulted in a 330-bp fragment and with primers II and IIIb, in a 260-bp fragment. The 330-bp fragment was isolated and used for probing a third-instar larval Lambda-Zap II cDNA library; a secondary screen was carried out with the 260-bp fragment. Nine secondary positive clones were identified and subcloned into Bluescript SK^+ via *in vivo* excision. Partial sequence (using PCR primer I) was obtained for all clones that proved to be identical over 200 bp downstream of primer I.

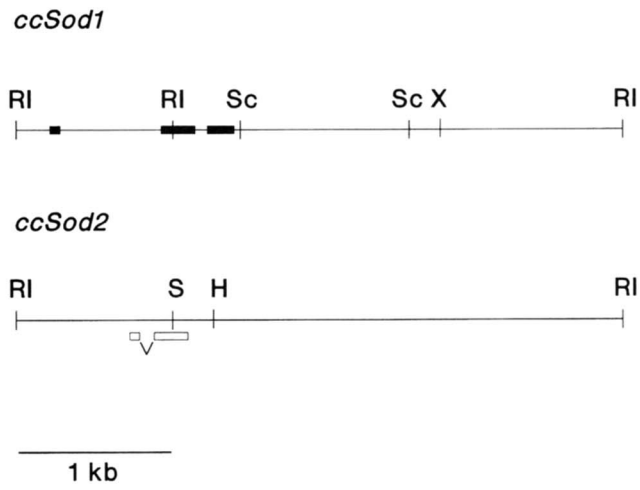

FIGURE 2.—Restriction maps of the *ccSod1* and *ccSod2* genes in *Ceratitis capitata*. The restriction map of *ccSod1*, with the exons shown as solid boxes, is taken from KWIATOWSKI *et al.* (1992). The probe for *ccSod1* was a 4-kb clone, whereas the probe for *ccSod2* was a 330-bp cDNA fragment generated by primers I and IIIb (shown on the figure as two open boxes). Restriction enzymes are R1, *Eco*RI; Sc, *Sca*I; X, *Xba*I; S, *Sal*I; H, *Hin*d III.

Figure 1 gives the nucleotide sequence of *ccSod2* cDNA, as well as the inferred amino acid sequence. The 453 nucleotide-long sequence codes for a 150 amino acid-long polypeptide. Two putative regulatory sequences are identified: a TATA box at position 121 and a polyadenylation signal at 1011.

We used the cDNA *ccSod2* to screen an Embl 3 genomic library and isolated the genomic homologue (*Sod2 gen*) for this gene. Figure 2 shows simplified restriction maps for the genomic clones of the two medfly *Sod* genes.

Amino acid comparisons: Figure 3 displays the alignment of nine Cu/Zn SOD amino acid sequences, six from insects including *ccSod1* and *ccSod2* and three from baculovirus, human, and fluke. Invariant in all enzymes compared are all essential amino acid residues of the active site (GETZOFF *et al.* 1989), including metal ligands (His-47, 49, 64, 72, 81, 122 and Asp-84) and two other residues (Arg-145 and Asp-126) involved in catalytic reaction, as well as the residues that determine the stability of the environment of the active site (Gly-45, 62, 83, 140, 143 and Pro-67). The disulphide bridge residues (Cys-58 and 148) are strictly conserved, whereas dimer-contact

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                                  20                  40                  60
D. melanogaster MVVKAVCVINGDA--KGTVF FEQESSGTPVKVSGEVCGLA KGLHGFHVHEFGDNTNGCMS
D. virilis      ..................... ....GE.C....T...T... ..Q..............
D. willistoni   ..................... ....DN.A....T...T..G .....................
Chymomyza       ..A................Y ....DACA....C..IT..N ..Q..............
Ceratitis SOD1  ............V......H ...QDAKS..L.T...N... ..................T.
Ceratitis SOD2  .--E.IAYVE.PV.V..N.T .I.NGCSEN.H.HVYLT..T P.K.......K..L..A.A.
Baculovirus     .--..I.I.S..-.VH.KIY .Q...ANQ.L.I..YLLN.P R.........Y..TS...T.
Human           A-T.....LK..GPVQ.IIN ...KE.NG....W.SIK..T E..............A..T.
Schistosoma     .--.....MT.T.GV..V.K .T..TDNG..H.HA.FS..K A.K..........T....T.

                                  80                  100                 120
D. melanogaster SGPHFNPVGKEHGAPVDENR HLGDLGNIEATG-DCPTKVN ITDSKITLFGADSIIGRTVV
D. virilis      ........Q......T.... ........I.N...G..P.. .C.C....L..N........
D. willistoni   .......HS......G.... ..........S..SG..A.. ........V..N........
Chymomyza       .......LN......T.... ..........P...G....C .N........EN..V.....
Ceratitis SOD1  A........NS....S.L.. ..........S...GA...E .S.KL.....EN..V...I.
Ceratitis SOD2  T.G....DKMD....G..V. .V........DA.NGVVDTT F..HL.S.T.KRT.V..GL.
Baculovirus     A.E....TNED....DA.I. .V......KSA.YNSL.E.. MM.NVMS.Y.PHN....SL.
Human           A......LSRK..G.K..E. .V.....VT.DK..GVAD.S .E..V.S.S.DHC.....L.
Schistosoma     A.A....TKQ.....E.SI. .V.....VV.GA..GNAVY. A..KL.S.N.SH......M.

                                  140                 156
D. melanogaster VHADADDLGQGGHELSKSTG NAGARIGCGVIGIAKV
D. virilis      ....P....K.......T.. ..............I
D. willistoni   ....P....K.......T.. ..............I
Chymomyza       ....P....K.......... ..............C.I
Ceratitis SOD1  ....P....K.......T.. .....L.......C.I
Ceratitis SOD2  ..ELT....K.C.PD..K.. ...G.LA.....V-.-
Baculovirus     ..T.K....LTD.P...T.. .S.G.L...I.A.C.-
Human           ..EK.....K..N.E.TK.. ...S.LA.......Q-
Schistosoma     I.ENE....R.......V.. ...G.LA.....L.AE
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FIGURE 3.—Amino acid sequence alignment of Cu,Zn SOD from flies, baculovirus, humans and a fluke. Residues identical with those in *D. melanogaster* are represented by dots; gaps in sequence alignments by dashes. Sequences are from the following sources: *D. melanogaster, D. virilis, Chymomyza amoena, C. capitata* SOD1 (KWIATOWSKI *et al.* 1992); *D. willistoni* (KWIATOWSKI *et al.* 1994); Baculovirus, *Autographa californica* (TOMALSKI *et al.* 1991); human (SHERMAN *et al.* 1983); fluke, *Schistosoma mansoni* (CORDEIRO DA SILVA *et al.* 1992).

amino acids located at positions 6, 8, 18, 51–55, 115–117 and 150–155 are conserved either absolutely or functionally in most enzymes. This conservation suggests that both medfly SOD are functional enzymes.

The average number of amino acid differences between the Drosophilidae Cu/Zn SOD and ccSOD1 is 30.8 ± 0.9, which is 20% of the total 153 amino acids (see Table 1). This degree of divergence (as well as the divergence among the Drosophilid species) corresponds well to the phylogenetic relationships between the medfly and the Drosophilids, thus indicating that *ccSod1* is orthologous with the Drosophilid *Cu/Zn Sod,* that is, the divergence among these SOD reflects speciation events.

A different picture emerges for *ccSod2,* which differs from the Drosophilid species by 68.4 ± 0.7 (46%) amino acids on the average. This is approximately the degree of differentiation between the Drosophilid SOD and those of baculovirus (44%), human (39%), and the fluke (38%). This indicates that *ccSod2* is paralogous to *ccSod1* and to the Drosophilid *Cu/Zn Sod,* that is, it probably arose by gene duplication in a rather distant ancestor.

Phylogenetic analysis: Figure 4 shows a phylogenetic tree obtained by the maximum parsimony method with bootstrap resampling (FELSENSTEIN 1985). The lengths of the branches reflect PAM differences (DAYHOFF 1978) between sequences. The tree includes a number of distant species, including vertebrates, plants, fungi and prokaryotes. Figure 4 shows a deep division between prokaryotes and eukaryotes. Among the eukaryotes a deep branch separates two extracellular enzymes (human and fluke) from all intracellular SOD. Yeast and plants appear as monophyletic groups (with fairly high incidence of bootstrap resampling, 89 and 65%, respectively). Within the animal branch, a fluke (Schistosoma) protein is undifferentiated, but there are two monophyletic groups, vertebrates (71%) and insects, including ccSOD1 (97%). The position of Ceratitis ccSOD2 is undefined with respect to dipteran ccSOD1 and the vertebrate, plant, fungal, and extracellular Cu,Zn SOD, which suggests that the duplication of this gene is very ancient.

Cytological location: The 1.6-kb cDNA clone for *ccSod2* maps to a site on chromosome *4L* at position 42B (see Figure 5A). However, there is also a faint signal repeatedly observed at position 36D on chromosome *3L* (see Figure 5B). This weak signal is not due to cross-

TABLE 1

Amino acid differences between animal Cu/Zn SOD enzymes

Species:	2	3	4	5	6	7	8	9
Species								
1 *D. melanogaster*	20	19	25	33	71	65	58	58
2 *D. virilis*		15	22	31	69	67	60	58
3 *D. willistoni*			21	30	67	66	58	58
4 *Chymomyza*				29	68	66	59	61
5 *Ceratitis* SOD1					67	66	60	59
6 *Ceratitis* SOD2						74	69	63
7 Baculovirus							72	71
8 Human								60
9 *Schistosoma mansoni*								

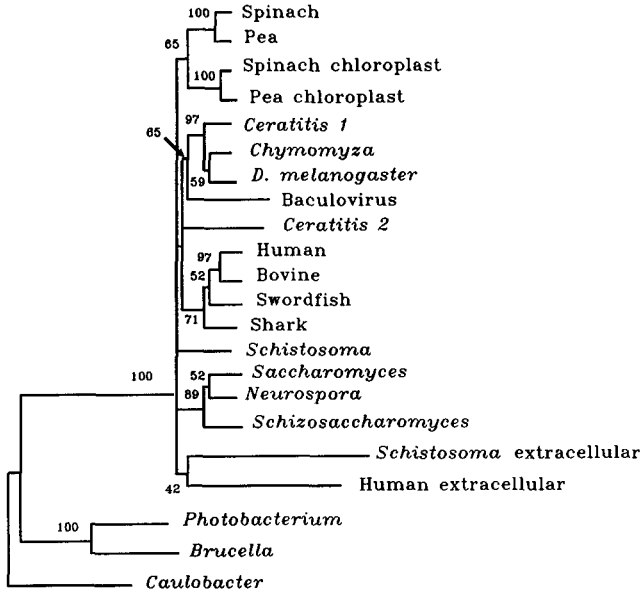

FIGURE 4.—Phylogenetic relationships of the Cu,Zn SOD. The topology of the consensus tree is obtained by maximum parsimony with bootstrap resampling, by consecutive execution of the Seqboot, Protpars and Consense programs of the PHYLIP 3.5 phylogenetic inference package (FELSENSTEIN 1989). The branch lengths of the tree are proportional to PAM distance values (DAYHOFF 1978) (obtained with the Protdist program of PHYLIP) and are calculated by running the FITCH program of PHYLIP with a consensus tree. The numbers at the forks indicate the bootstrapped number of times the group encompassing all species to the right occurs out of 100 samplings (the number has been omitted in three places where it is smaller than 50). Sequences are obtained by a Gopher service from databases maintained at IUBio archives at Indiana University: GenBank (Baculovirus, M68862; Bovine, X54799; Caulobacter, M55259; Ceratitis, M76975; Chymomyza, X61687; *Drosophila melanogaster*, X13780; Human, K00065; Human extracellular, J02947; Neurospora, M38181; Pea, M63003; Pea chloroplast, J04087; Photobacterium, J02658; Saccharomyces J032279; Schizosaccharomyces, X66722; Schistosoma M97298; Schistosoma extracellular, M27529; Spinach, X53872; Spinach chloroplast, D10244; and Swiss-Protein (Brucella, P15453; Shark, P11418; Swordfish, P03946). The alignment of the 22 sequences produced by ClustalV program (HIGGINS *et al.* 1992) is available upon request from the authors (J.K. or F.J.A.).

hybridization with *ccSod1*, because the 4-kb genomic clone of *ccSod1* maps exclusively to chromosome *6* at position 99A (Figure 5C).

DISCUSSION

This is the first case reported of two Cu/Zn *Sod* genes that have been cloned and sequenced from an insect. The isolation of two different *Sod* genes confirms preliminary biochemical evidence suggesting the presence of two *Sod* genes in the medfly (FERNANDEZ-SOUSA and MICHELSON 1976).

It is pertinent to explain how and perhaps why two different gene sequences were isolated. *ccSod1* was cloned from a genomic medfly library by cross-hybridization to a *D. melanogaster* cDNA probe. All positive clones contained the same *Sod* gene sequence. *ccSod2* was isolated from a medfly third-instar cDNA library, using as a probe a PCR fragment amplified from adult cDNA. Thus, the cloning of *ccSod1* was dependent on the degree of homology with the *D. melanogaster* counterpart, whereas the cloning of *ccSod2* was dependent on the specificity of the PCR primers, as well as the level of expression of *Sod* in adults and third-instar larvae.

The two genes *ccSod1* and *ccSod2* are different to the extent that they do not cross-hybridize even at low to moderate stringency on a library screen, genomic Southern blot, or *in situ* hybridization to polytene chromosomes (data not shown). This might explain why only *ccSod1* sequences were isolated from a genomic library when the homologous gene from *D. melanogaster* was the probe and, similarly, why only *ccSod2* sequences from the cDNA library showed as positive clones when a *ccSod2* fragment was used as a probe.

Differences in gene expression through development may have also played a role. To cover the 3′ end (aa147–152) of the gene, we designed two primers, IIIa and IIIb, that differ at position 147 in that IIIb assumes alanine (the amino acid present in nondipteran Cu/Zn SOD) and IIIa assumes glycine (the amino acid present in Drosophila SOD). IIIb was effective, in conjunction with either primer I or II, for PCR amplification from adult cDNA; IIIa failed with both 5′-end primers. Because we know that *ccSod1* codes for glycine at position 147, it would seem to follow that this gene is likely to be expressed in the adult medfly only at low levels or not at all. Consistent with this tentative inference, preliminary Northern blot analysis indicates that *ccSod1* is expressed mainly in early embryo rather than later developmental stages. GRAF and AYALA (1986) have shown, on the basis of CRM assays, that in *D. melanogaster* SOD content is low in young adults but it gradually increases after emergence from the pupa, so that by the seventh day SOD levels are two to three times higher than just after emergence. SOD levels are also high in embryos but low through the larval and pupal stages. Using the same RNA blots, stripped and reprobed, *ccSod2* shows hybridization signal from third-instar larvae through adult but not in the early developmental stages.

The inferred amino acid sequences show that the coding region for *ccSod1* is three amino acids longer than that of *ccSod2*. Nevertheless, it would seem that both genes encode functional Cu/Zn SOD because all the essential amino acid residues of the active site are strictly conserved, including those involved in metal ligand binding, catalytic reaction, and stability of the active site. The lack of a signal peptide in either gene suggests both genes may have a cytoplasmic function. The human Cu/Zn SOD is localized in the peroxisomes in fibroblasts and hepatoma cells (KELLER *et al.* 1991), and this locale may be imposed by the putative peroxisome-targeting tripeptide Ser-Arg-Leu (Figure 3, positions 144–146) near the c-terminal of the polypeptide.

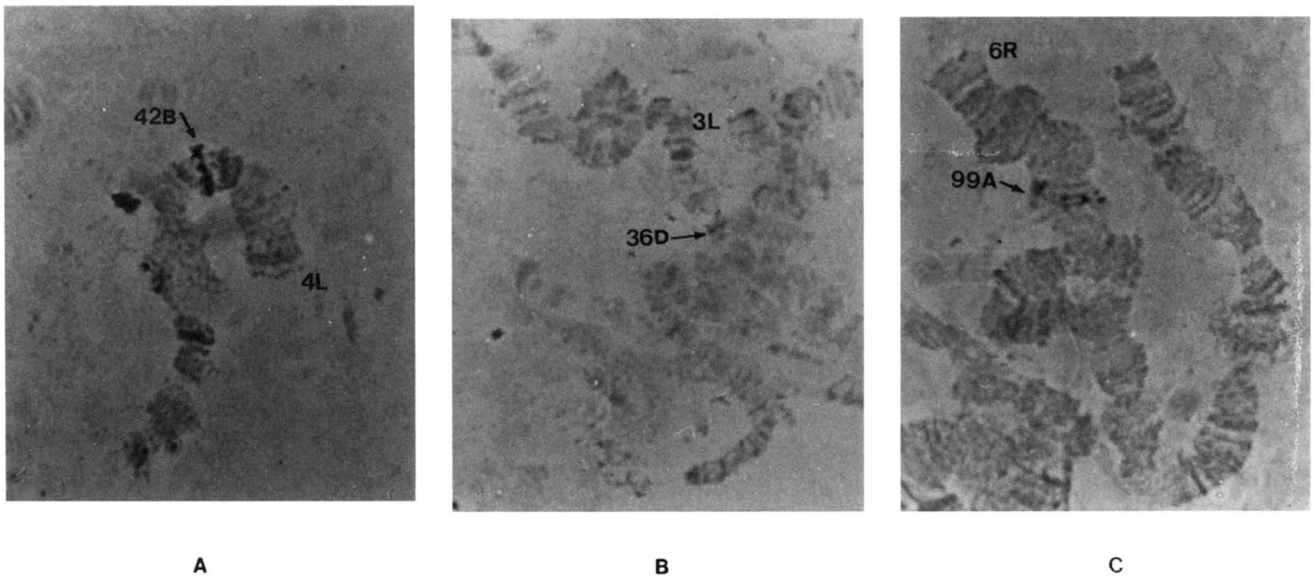

FIGURE 5.—*In situ* hybridization of the Ceratitis polytene chromosomes with *ccSod2* (A and B) and *ccSod1* (C). The *ccSod2* probe is a 1.6-kb full-length cDNA clone; *ccSod1* is a 4-kb genomic clone. B shows a faint hybridization signal at 36D consistently found with the *ccSod2* probe.

ccSOD1 also has the canonical tripeptide Ala-Arg-Leu (GOULD *et al.* 1989) in positions 144–146. But neither of these tripeptides occurs in ccSOD2, which has Gly-Arg-Leu instead, which suggests the subcellular locale may be different for ccSOD1 and ccSOD2.

There is some anecdotal evidence, both cytological and molecular, that the medfly is particularly susceptible to duplication events. For example, tandem duplications are frequently detected on polytene chromosomes (A. ZACHAROPOULOU, personal communication), and at least two molecularly characterized gene systems, vitellogenin (RINA and SAVAKIS 1991) and *Adh* (C. SAVAKIS, personal communication), include duplications of functional genes. However, the presence of two Cu/Zn *Sod* genes in Ceratitis is unlikely to be a result from a recent tandem duplication because the two genes are located on different chromosomes. Moreover, *ccSod2* has an intron at position aa67/68, whereas *ccSod1* has an intron at aa95/96, 28 amino acids away (data not shown). An intron similarly located as in *ccSod2* occurs in a gene coding for extracellular SOD in the fluke, *Schistosoma mansoni* (SIMURDA *et al.* 1988).

Evidence against a recent duplication derives also from the degree of divergence between ccSOD1 and ccSOD2 (Table 1). The difference amounts to 67 amino acids, approximately as large as between human SOD and either ccSOD1 (60 amino acids) or ccSOD2 (69 amino acids). In addition, the average number of amino acid differences compared with Drosophila SOD is 30.8 ± 0.9 for ccSOD1 but 68.4 ± 0.7 for ccSOD2. The possibility that ccSOD2 may have evolved at a much faster rate than ccSOD1 after a recent duplication seems unlikely in view of the large differentiation between ccSOD1 and ccSOD2. Furthermore, such rapid evolution of ccSOD2 (accounting for 38 additional amino acid differences) should have similarly increased the degree of differentiation of ccSOD2 relative to human and Schistosoma SOD, which are only slightly or not at all more different from ccSOD2 (69 and 63 amino acid differences, respectively) than from ccSOD1 (59 and 63 differences, respectively).

These results point out an ancient duplication of *ccSod1* and *ccSod2*. The fact that ccSOD2 is as different from ccSOD1 as it is from human and Schistosoma SOD suggests that the duplication is about as old as the divergence of the phyla chordata and either the arthropoda or platyhelminthes phyla, perhaps as old as the beginning of the Cambrian period, some 590 million years ago. The inference that the duplication is ancient assumes that SOD has evolved at an approximately constant rate. The assumption of an SOD molecular clock has been challenged (AYALA 1986), but it has been recently shown that, under certain reasonable assumptions, SOD behaves as a fairly accurate molecular clock to the extent that similar numbers of amino acid differences reflect similar spans of time since divergence (FITCH and AYALA 1994). The conclusion is that the duplication of *ccSod1* and *ccSod2* is ancient and may be as old, or nearly, as the emergence of the arthropod phylum.

If the *ccSod1*/*ccSod2* duplication is ancient, two hypotheses are possible. The first hypothesis is that *ccSod2* has been transmitted vertically in the *Ceratitis* lineage since it first appeared by duplication. If so, it follows that *ccSod2* would have been inherited in other dipteran (and even arthropod) lineages. Unless it became lost in all other lineages, *ccSod2* should be present in other dipterans as well. The second hypothesis is that *ccSod2* became incorporated in the Ceratitis lineage by horizontal gene transfer. There is a baculovirus homo-

logue of *Sod* (TOMALSKI *et al.* 1991), which the virus may have acquired from an animal species, possibly a lepidopteran host. Horizontal transfer of repetitive DNA sequences (*P* elements) between Drosophila species has been demonstrated (DANIELS *et al.* 1990). Horizontal transfer of autologous protein-coding genes between eukaryotes has not yet been discovered, but its possibility cannot be convincingly excluded. However, if horizontal transfer from some other species to Ceratitis would have occurred, it still follows that *ccSod2* would be present in lineages other than the donor's, unless it would have been lost in all of them.

Evidence exists of other ancient *Sod* duplications (*e.g.*, KWIATOWSKI *et al.* 1991; SMITH and DOOLITTLE 1992; FITCH and AYALA 1994). For example, a duplicated gene coding for an extracellular form of the enzyme exists in rats and humans that is ostensibly as old, or older than, the divergence of the animal, plant, and fungi kingdoms. Ancient duplications, although not quite as old, are present in *C. elegans* and *S. mansoni*. All higher plants studied (including scots pine, garden pea, spinach, and tomato) share an *Sod* duplication, yielding a chloroplast and a cytoplasmic form, that predates the divergence of gymnosperms and angiosperms.

SOD constitutes a diverse class of enzymes with Cu/Zn SOD being only part of it. Early eukaryotes certainly required many tools to cope with the harmful effects of toxic oxygen species in various subcellular compartments. Consequently, it seems likely that early eukaryote life entailed the appearance of a battery of SOD to meet this challenge. The available evidence suggest that ccSOD2 represents a new class of Cu/Zn SOD, but it remains to be seen whether it is specific to animals, insects, or any other taxa. We are currently searching for a *ccSod2* counterpart in other insects. In addition, a third site on chromosome *3*, appearing as a second weak signal in *in situ* chromosome hybridization with *ccSod2*, raises the possibility that there is a third *Sod* gene or possibly a pseudogene or *Sod*-like sequence.

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