Mammalian antioxidant protein complements alkylhydroperoxide reductase (*ahpC*) mutation in *Escherichia coli*

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The *MER5* [now called the *Aop1* (antioxidant protein 1) gene] was cloned as a transiently expressed gene of murine erythroleukaemia (MEL) cell differentiation and its antisense expression inhibited differentiation of MEL cells. We found that the *Aop1* gene shows significant nucleotide sequence similarity to the gene coding for the C22 subunit of *Salmonella typhimurium* alkylhydroperoxide reductase, which is also found in other bacteria, suggesting it functions as an antioxidant protein. Expression of the *Aop1* gene product in *E. coli* deficient in the C22-subunit gene rescued resistance of the bacteria to alkylhydroperoxide. The human and mouse *Aop1* genes are highly conserved, and they mapped to the regions syntenic between mouse and human chromosomes. Sequence comparisons with recently cloned mammalian *Aop1* homologues suggest that these genes consist of a family that is responsible for regulation of cellular proliferation, differentiation and antioxidant functions.

INTRODUCTION

The *MER5* cDNA, which encoded a 257-amino-acid protein showing no sequence identity with other known proteins, was cloned from RNA preferentially synthesized in murine erythroleukaemia (MEL) cells during the early period of MEL cell differentiation [1]. To understand the role of the *MER5* gene in the differentiation, we have transferred the *MER5* cDNA into MEL cells in both sense and antisense orientation. Only in the transformants with the antisense *MER5* cDNA, did their elevated expression inhibit differentiation, suggesting that the *MER5* gene product may promote early events in the differentiation of MEL cells [2]. However, its action mechanism has not been described. After we had published the results of that study, the sequence of a C22 subunit of bacterial alkylhydroperoxide reductase was reported [3,4] and we found that the bacterial gene shows 38% identity with the *MER5* gene. Thus we attempted to examine whether the *MER5* gene product has the same function as the bacterial gene product. In the present study we report that the *MER5* gene can complement bacterial mutation and that its product is a new type of mammalian antioxidant protein.

MATERIALS AND METHODS

Bacterial mutants and construction of the *MER5* gene mutants

ahpd5 (TA4315) and pGS01 were kindly provided by Dr. Gisela Storz (National Institutes of Health, Bethesda, MD, U.S.A.). *ahpd5* (TA4315) is an *E.coli* K12 strain carrying a deletion of the entire alkylhydroperoxide reductase (*ahp*) locus. Plasmid pGS01 is a derivative of pAQ27 [2,7] carrying sequence of alkylhydroperoxide reductase component F52a. *ahpd5*/pGS01 was used for the host cell of the following experiments. Mutants were prepared by site-specific mutagenesis [5]. For the $Cys^{109} \rightarrow Gly$ mutation (*Cys1*), oligonucleotide 5′-AACAATTTCTGTAGGGCCCAC-AAATGTGAAATC-3′ was used. For the $Cys^{230} \rightarrow Gly$ mutation (*Cys2*), 5′-TGTCCAGTTGGCTGGGCCCACTTCTTCATG-GGT-3′ was used.

Complementation of the *aphC* mutation with the *MER5* gene in *E. coli*

The bacteria containing sense and antisense and mutated *MER5* genes were grown in the presence of isopropyl β-D-(−)-thiogalactopyranoside (IPTG) (1 mM), which induced the *MER5* gene products, to their saturation density (1×10^8 cells/ml). The cell suspensions (0.1 ml; about 1×10^8 cells/ml) were transferred to 96-well plates and peroxide compounds (0.1 ml) were added to the wells. Cumene hydroperoxide (Aldrich), t-butyl hydroperoxide (Sigma) and H_2O_2 (Santoku Chemical Industries, Tokyo, Japan) were used as hydroperoxides. After incubation for 20 min, the cell suspensions were serially diluted with Terrific broth and seeded on the agar plates. After 1 day's incubation at 37 °C, the numbers of colonies were scored.

Detection of the *MER5* gene product in *E. coli*

The cell suspensions were centrifuged, and the pellets were resuspended in SDS sample buffer [125 mM Tris buffer (pH 6.8)/20% glycerol/4% SDS/1.4 M 2-mercaptoethanol]. The cell suspensions were lysed on sonication. The lysates (10 μg of protein) were separated by 0.1%-SDS/12%-PAGE, and the proteins were blotted on to a nitrocellulose membrane [CELLULOSENITRAT(E); S&S, Dassel, Germany]. The membrane was stained with the *MER5* antiserum [2] and bands were revealed with ECL Western blotting detection system (Amersham).

Cloning of the human *MER5* gene

A human erythroid cell line (YN-1-0-A) cDNA library in Okayama–Berg vector were kindly provided by Dr. Shigeki Shibahara (Tohoku University, Sendai, Japan). To obtain the

Abbreviations used: *ahp*, alkylhydroperoxide reductase locus; MEL, murine erythroleukaemia; IPTG, isopropyl β-D-(−)-thiogalactopyranoside; RFLP, restriction-fragment-length polymorphism; *Bpag2*, bullous pemphigoid antigen 2; *Csfgmra*, colony-stimulating-factor granulocyte macrophage receptor alpha chain; ORF, open reading frame; SOD, superoxide dismutase; Aop1, antioxidant protein 1 (*Aop1* is the gene coding for it); *PAG*, human proliferation-associated gene; TSA, thiol-specific-antioxidant (*TSA* is its gene).

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Table 1 Comparison of the sequence of the *MER5* product with those of the protein encoded by *ahpC* (C22) and other proteins.

The alignment was carried out using Genetyx version 6. The sequence similarity to the *MER5* product shows the most matched alignment between MER5 and each gene product. Seven amino acid residues of cysteine-containing conserved regions (1 and 2) are shown.

Gene	Source of gene	Function	Sequence identity with the MER5 product (%)	Sequence		Reference
				Region 1	Region 2	
C22	*S. typhimurium*	Alkylhydroperoxide reductase	38.0	TFVCPTE	GEVCPAK	[3,4]
AhpA	*B. subtilis*	Unknown	56.3	TFVCPTE		P. Zuber, personal communication
26 kDa	*H. pylori*	Unknown	48.9	TFVCPTE	GEVCPAG	[16]
ORF3	*C. pasteurianum*	Rubredoxin	54.4	TFVCPTE	GGMCALD	[15]
TSA	*S. cerivisiae*	TSA	58.2	TFVCPTE	VTVLPCN	[20]
29 kDa	*E. histolytica*	Unknown (surface antigen)	47.2	TFVCPTE	GAVCPLN	[19]
Arch	*M. thermoauto trophicum*	Unknown	30.7	TFVCTTE	GVAAPAN	[14]

human *MER5* cDNA clone by the colony-hybridization method, *E. coli* containing cDNAs were seeded on a Luria–Bertani plate and the colonies formed were transferred on to nylon filters (Colony/Plaque Screen, du Pont). Filters containing 1×10^4 colonies/sheet were hybridized with a ^{32}P-labelled 798-bp *Pvu*II fragment containing almost-full-length *MER5* cDNA [1]. Seven colonies were finally obtained from about 5×10^5 colonies of the library. All clones had the same length of cDNA (1.4 kb); thus one was selected and the cDNA fragment was converted into the Bluescript plasmid vector by a DNA Ligation Kit (Takara Shuzo, Kyoto, Japan). The sequences of both strands of the selected clone were determined using an M13 Sequencing Kit (Toyobo co., Tokyo, Japan). Recording and analysis of the sequence were performed using the Genetyx and Genetyx-CD (Software Development Co.,Tokyo, Japan).

Mouse chromosome mapping by the interspecific back-cross

The interspecific back-cross mapping panel has been typed for over 1500 loci that are well distributed among all the autosomes as well as the X chromosome [6]. C57BL/6J and *spretus* DNAs were digested with several restriction enzymes and analysed by Southern-blot hybridization for informative restriction-fragment-length polymorphisms (RFLPs) using a mouse cDNA *Aop1* probe. A 7.6 kb *M. spretus Eco*RI RFLP was used to follow the segregation of the *Aop1* locus in back-cross mice. The mapping results indicated that *Aop1* is located in the distal region of the mouse chromosome 19 linked to bullous pemphigoid antigen 2 (*Bpag2*) and colony-stimulating-factor granulocyte macrophage receptor alpha chain (*Csfgmra*). Although 133 mice were analysed for every marker and are shown in the segregation analysis, up to 166 mice were typed for some pairs of markers. Each locus was analysed in pairwise combinations for recombination frequencies using the additional data. The ratio of the total number of mice exhibiting recombinant chromosomes to the total number of mice analysed for each pair of loci and the most likely gene order are: centromere–*Bpag2*–16/146–*Aop1*–3/166–*Csfgmra*. The recombination frequencies [expressed as genetic distances in centimorgans (cM) ± S.E.M.] are: –*Bpag2*–11.0 ± 3.0–*Aop1*–1.8 ± 1.0–Csfgmra. For RFLP analysis, DNA isolation, restriction-enzyme digestion, agarose-gel electrophoresis, Southern-blot transfer and hybridization were performed essentially as described [7]. All blots were prepared with Zetabind nylon membrane (AMF-Cuno). The probe, a 1382 bp mouse cDNA clone, was labelled with [α-^{32}P]dCTP using a nick-translation labelling kit (Boehringer-Mannheim); washing was done to a final stringency of 0.8 × SSCP (96 mM NaCl/12 mM sodium citrate/12 mM Na_2HPO_4/4 mM NaH_2PO_4)/0.1 % SDS at 65 °C. A major fragment of 8.8 kb was detected in the *Eco*RI-digested C57BL/6J DNA, and a major fragment of 7.6 kb was detected in *Eco*RI-digested *M. spretus* DNA. The presence or absence of the 7.6 kb *M. spretus*-specific *Eco*RI fragment was monitored in back-cross mice. A description of the probes and RFLPs for the loci linked to the *Aop1*, including *Bpag2* and *Csfgmra*, has been reported previously [8,9]. Recombination distances were calculated as described [10] using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Human chromosome mapping by *in situ* hybridization

Fluorescence *in situ* hybridization was carried out with a biotinylated cDNA probe. *A Pst*I–*Xba*I fragment (1318 bp), containing almost the entire coding region of the human *Aop1* gene, was labelled with biotin-16 -dUTP by nick translation and used for *in situ* chromosome mapping by the FISH method described by Takeda et al. [11].

RESULTS AND DISCUSSION

The *MER5* gene shows significant nucleotide sequence identity with bacterial genes

Recent 'homology' searches showed that the *MER5* gene product has a significant amino acid sequence similarity to the C22 subunit of bacterial (*Salmonella typhimurium*) alkylhydroperoxide reductase [3,4] and products of other bacterial genes, as shown in Table 1. *Salmonella* alkylhydroperoxide reductase consists of two subunits, C22 and F52a; F52a shows strong sequence similarity to thioredoxin reductase [4]. This operon consisting of *C22* and *F52a* genes is induced by H_2O_2 under the regulation of *OxyR* [12]. The sequence similarity between the *Salmonella* C22 subunit (amino acid residues 11–170) and the *MER5* product (amino acid residues 73–234) was 38.0 %, and

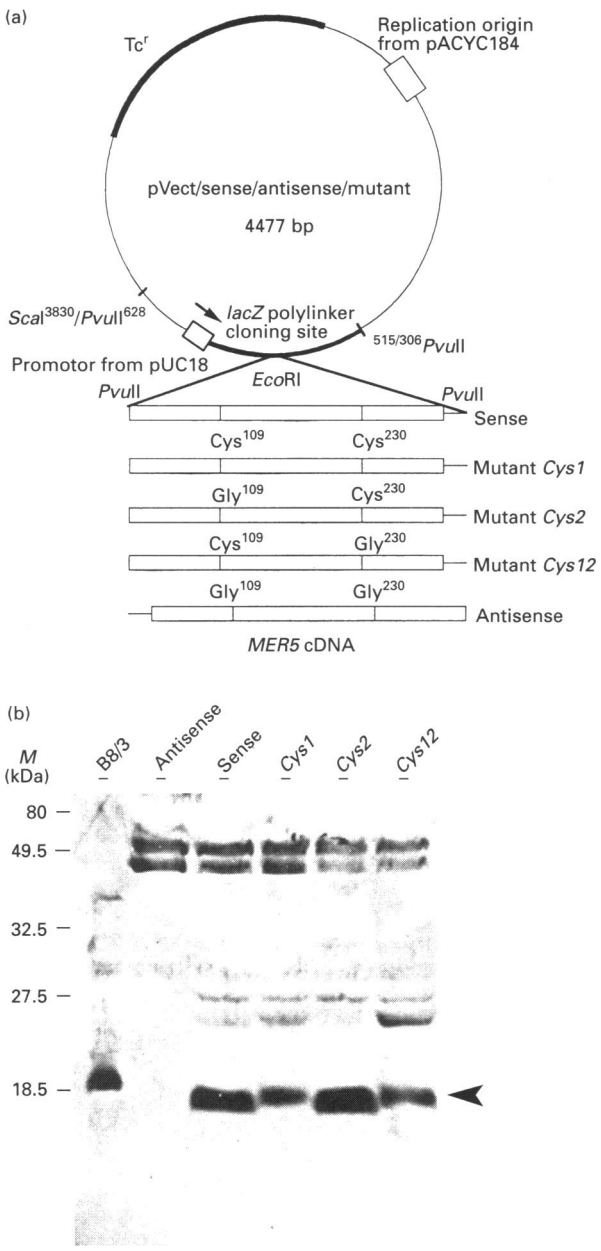

Figure 1 Introduction and expression of the *MER5* genes in *E. coli* lacking the C22 subunit of bacterial alkyl hydroperoxide reductase

(**a**) Plasmid construction of *MER5* and its mutant genes in the pVect plasmids. pVect is a derivative of pACYC184 and pUC18 carrying sequences between the *PvuII*^{515} (pACYC184)–*PvuII*^{306} (pUC18) and *ScaI*^{3830} (pACYC184)–*PvuII*^{628} (pUC18) sites. *MER5* cDNA fragment (*PvuII*–*PvuII* 798 bp) is ligated to the *EcoRI* site of the *lacZ* polylinker site and expressed by the *lac* promoter from pUC18. (**b**) Expression of transfected *MER5* and its mutant genes in *E. coli*. Western blotting was performed to detect the expression of transfected *MER5* cDNAs. Abbreviations: *M*, molecular mass; Tc^r, tetracyclin resistance.

the region near the cysteine residue showed high similarity among eight different genes. The *Bacillus subtilis ahpA* gene product, which might have a similar function to the C22 protein of *Salmonella*, showed a stronger similarity (P. Zuber, personal communication). The open reading frame (ORF) of the nucleotide sequence linked to the superoxide dismutase (SOD) gene of the archaebacterium *Methanobacterium thermoautotrophicum* shows considerable similarity to the *MER5* gene [14]. It is noteworthy that the archaebacterium C22 homologue is linked to the SOD gene rather than thioredoxin reductase gene. ORF3 is linked to the gene for *Clostridium pasteurianum* rubredoxin, which is one of the iron–sulphur proteins present in a variety of anaerobic bacteria and considered as an electron carrier in the oxidation system of fatty acids and hydrocarbons [15]. Since ORF3 is linked to ORF1, which shows similarity to the F52a-component, the alkyl hydroperoxide reductase gene is linked to rubredoxin gene in *C. pasteurianum*. *Helicobacter pylori* 26 kDa protein was identified from a gene encoding a species-specific protein of the bacterium [16].

Complementation of alkylhydroperoxide reductase (*aphC*) mutation in *E. coli*

Because of a striking conservation of structure of the *MER5* gene with the bacterial genes, we examined whether this gene can complement the deficiency of the C22-subunit gene in *E.coli*. By introducing the plasmid pGS01, which deletes entire *ahp* locus, the bacterium (ahpd5/pGS01) with only the *ahpC* locus was generated [5]. Then, the *MER5* gene that had been linked to the *lac* promoter in sense and antisense orientation, was introduced into *E.coli* (ahpd5/pGS01) (Figure 1a). The bacteria were grown in the presence of IPTG (1 mM) to induce the *MER5* gene product, and the expression of the *MER5* gene product was confirmed by the Western-blot analysis using the antiserum generated for an oligopeptide deduced from the *MER5* gene sequence [2] (Figure 1b). The resistance of the bacteria to alkylhydroperoxide was monitored by their viability (Table 2). A 10–50-fold increase in resistance to cumene hydroperoxide was obtained in the bacteria expressing the *MER5* gene product. The bacteria also acquired resistance to other hydroperoxides, such as H_2O_2 and t-butyl hydroperoxide. The resistance to H_2O_2 was especially high. These results indicate that the *MER5* gene product can complement the activity of the C22 subunit of bacterial alkylhydroperoxide reductase. Since the conserved regions of the *MER5* gene contain cysteine residues and the functional importance of two cysteine residues has been suggested [3], we mutated two cysteine residues (Figure 1a) and determined whether these mutations had lost their *MER5* function. The results clearly showed that *Cys1* and *Cys12* mutants completely lack activity, while the effect of the *Cys2* mutation seems less than the other two mutations (Table 2). These studies suggest that the Cys^{109} residue of *MER5* is very important for the complementation of the C22 subunit of bacterial alkylhydroperoxide reductase in *E.coli*.

Chromosome mapping of human and mouse *Aop1* (*MER5*) genes

Because of a striking conservation of structure and function of the *MER5* gene with the bacterial antioxidant protein genes, we called the *MER5* gene product Aop1 (antioxidant protein 1) and examined gene conservation in the genomes of Man (*Homo sapiens*), chicken (*Gallus gallus domestica*), snapping turtle (*Trionyx sinensis*) and fruitfly (*Drosophila melanogaster*) by Southern-blot hybridization and demonstrated that these organisms contained regions of sequence identity (results not shown). Next, we compared its sequence similarity with the human gene by isolating it from a cDNA library of a human leukaemia cell line. The sequence similarity between the cloned human gene (256 amino acid residues) and the mouse gene (257 residues) was 86% (see Table 3). The chromosomal location of mouse Aop1 was determined by inter-specific back-cross analysis by using progeny derived from the mating of (C57BL/6J × *M. spretus*) F1 mice with C57BL/6J mice [6–10]. The mapping

Table 2 Complementation of peroxidase resistance of the bacteria lacking the C22 subunit of alkylhydroperoxide reductase by the *MER5* gene products

0.2 μM Cumene hydroperoxide (a), 1 μM t-butyl hydroperoxide (b) and 40 μM H_2O_2 (c) were used for the experiments. Peroxide concentrations were determined at the points showing 0.1% viability of antisense-transfected *E. coli*.

			Survival of bacteria (%)				
Peroxide	Expt.	Mutant...	Sense	Antisense	*Cys1*	*Cys2*	*Cys12*
(a)	1		4.13 ± 0.03	0.25 ± 0.03	0.21 ± 0.03	0.97 ± 0.25	0.20 ± 0.03
	2		2.50 ± 0.19	0.21 ± 0.05	0.24 ± 0.00	0.34 ± 0.32	0.19 ± 0.02
	3		2.57 ± 0.18	0.16 ± 0.01	0.06 ± 0.00	0.33 ± 0.19	0.13 ± 0.03
(b)	1		2.32 ± 0.05	0.11 ± 0.04	0.54 ± 0.12	0.75 ± 0.14	0.37 ± 0.03
	2		1.80 ± 0.03	0.11 ± 0.04	0.18 ± 0.06	1.44 ± 0.21	0.51 ± 0.07
	3		1.83 ± 0.21	0.14 ± 0.02	0.24 ± 0.06	1.01 ± 0.07	0.43 ± 0.05
(c)	1		84.7 ± 1.2	1.5 ± 0.4			
	2		80.2 ± 6.5	0.3 ± 0.1			
	3		75.1 ± 6.9	0.6 ± 0.2			

Table 3 Comparison of the amino acid sequences of the *Aop1* gene family from mouse and Man

The alignment was carried out using Genetyx version 6. Sequence similarity to the *MER5* gene product and seven amino acid residues of the cysteine-containing conserved regions (1 and 2) are shown.

Gene	Function	Sequence similarity to the *MER5* product (%)	Region 1	Region 2	Reference
Aop1 (human)	Unknown	86.0	TFVCPTE	GEVCPAN	
MSP23 (mouse)	Stress protein	64.0	TFVCPTE	GEVCPAG	[18]
PAG (human)	Unknown (proliferation-associated)	64.7	TFVCPTE	GEVCPAG	[17]

results indicated that *Aop1* is located in the distal region of the mouse chromosome 19 linked to *Bpag2* and *Csfgmra* as shown in Figure 2. The distal region of mouse chromosome 19 shares a region of sequence identity with human chromosome 10q and the pseudautosomal region of the X and Y chromosomes (summarized in Figure 2). Chromosomal localization of human *Aop1* conducted by *in situ* hybridization [11] showed that the human *Aop1* gene is located at 10q25–26, as shown in Figure 2. Thus both results are consistent with the location of the *Aop1* gene in human and mouse chromosomes.

Aop genes consist of a family that is responsible for proliferation, differentiation and antioxidant function

After cloning of human *Aop1* gene, we identified the sequence similarity to other mammalian genes (Table 3). Human proliferation-associated gene (*PAG*), which codes for a 22 kDa protein, shows 64.7% identity with the *Aop1* gene [17]. Therefore our human *Aop1* gene is not identical with *PAG*. MSP23 was cloned as a 23 kDa stress-induced mouse peritoneal macrophage protein [18]. It is noteworthy that MSP23 shows more similarity to the *PAG* than the *Aop1* product, and these two gene products are about 60 amino acids shorter than *Aop1* and human *Aop1* gene products. The sequence similarity suggests that the *PAG* product is a human homologue of MSP23. *Aop1*, *PAG* and the MSP23 gene may consist of a family. It would be interesting to know whether more family members exist and whether these genes co-localize as a cluster within the same chromosomal region.

In addition, we found significant sequence identity with genes of two pathogenic human parasites. *Entamoeba histolytica* 29 kDa surface antigen protein shows significant identity with the *Aop1* [19]. *E. histolytica* is a pathogenic protozoan causing extensive mortality and morbidity worldwide through diarrhoeal disease and organ abscess formation. The surface antigen may play an integral role in the modulation of host–parasite interactions. *H. pylori* 26 kDa protein [16] also showed significant sequence identity. *H. pylori*, a prevalent human-specific pathogen, is a causative agent in chronic active gastritis, gastric and duodenal ulcers and gastric adenocarcinoma, one of the most common forms of cancer in humans. It would be worthwhile to examine whether these proteins have an antioxidant function and are involved in pathogenesis.

In *Saccharomyces cerevisiae* (baker's yeast), a thiol-specific antioxidant (*TSA*) gene was isolated and a *tsa* mutant, generated by homologous recombination, was produced. It was shown that the *TSA* product is not essential for cell viability under aerobic conditions, but under anaerobic conditions, especially in the presence of Methyl Viologen or a peroxide such as t-butyl peroxide and H_2O_2, the *tsa* mutant showed slow growth, suggesting that TSA acts as an antioxidant [20]. The sequence similarity between the *Aop1* genes and *TSA* suggests that these genes function in an oxidation–reduction system. It would be worthwhile to test whether the *Aop1* genes can complement the

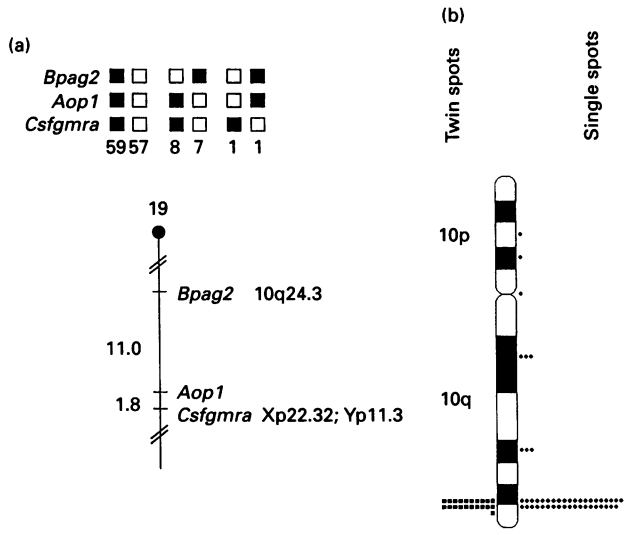

Figure 2 Chromosome localization of human and mouse *Aop1*

(**a**) *Aop1* maps in the distal region of mouse chromosome 19. *Aop1* was placed on mouse chromosome 19 by interspecific back-cross analysis. The segregation patterns of *Aop1* and flanking genes in 133 back-cross animals that were typed for all loci are shown at the top of the Figure. For individual pairs of loci, more than 133 animals were typed. Each column represents the chromosome identified in the back-cross progeny that was inherited from the (C57BL/6J × *M. spretus*) F_1 parent. The black boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of the *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 19 linkage map showing the location of *Aop1* in relation to linked genes is shown at the bottom of the Figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for human map positions of loci cited in the present study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by the William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD, U.S.A.). (**b**) *In situ* localization of human *Aop1* gene on chromosome 10q25-26. Among 550 cells, 60 signals (single and twin spots in the Figure) were found on 10q25-26 [statistical significance (P) < 0.01].

tsa mutant of yeast. Perhaps because the *Aop1* gene was isolated as differentiation-related gene [1,2], proliferation-associated gene (*PAG*) [17] and stress-induced proteins (MSP23 and TSA) [18,20], it is likely that redox regulation may be involved in the regulation of proliferation and differentiation. In fact, we have shown previously that the *Aop1* gene product enhances differentiation [2]. The *Aop1* gene products may be a new type of protein involved in regulation of proliferation/differentiation and antioxidant functions through redox regulation in mammalian cells.

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