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Differential developmental expression and cell type specificity of *Dictyostelium* catalases and their response to oxidative stress and UV-light

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Abstract

Cells of *Dictyostelium discoideum* are highly resistant to DNA damaging agents such as UV-light, γ-radiation and chemicals. The genes encoding nucleotide excision repair (NER) and base excision repair (BER) enzymes are rapidly upregulated in response to UV-irradiation and DNA-damaging chemicals, suggesting that this is at least partially responsible for the resistance of this organism to these agents. Although *Dictyostelium* is also unusually resistant to high concentrations of H_2O_2, little is known about the response of this organism to oxidative stress. To determine if transcriptional upregulation is a common mechanism for responding to DNA-damaging agents, we have studied the *Dictyostelium* catalase and Cu/Zn superoxide dismutase antioxidant enzymes. We show that there are two catalase genes and that each is differentially regulated both temporally and spatially during multicellular development. The *cat*A gene is expressed throughout growth and development and its corresponding enzyme is maintained at a steady level. In contrast, the *cat*B gene encodes a larger protein and is only expressed during the final stages of morphogenesis. Cell type fractionation showed that the CatB enzyme is exclusively localized to the prespore cells and the CatA enzyme is found exclusively in the prestalk cells. Each enzyme has a different subcellular localization. The unique developmental timing and cell type distribution suggest that the role for *cat*B in cell differentiation is to protect the dormant spores from oxidative damage. We found that exposure to H_2O_2 does not result in the induction of the catalase, superoxide dismutase, NER or BER mRNAs. A mutant with greatly reduced levels of *cat*A mRNA and enzyme has greatly increased sensitivity to H_2O_2 but normal sensitivity to UV. These results indicate that the natural resistance to oxidative stress is not due to an ability to rapidly raise the level of antioxidant or DNA repair enzymes and that the response to UV-light is independent from the response to reactive oxygen compounds.

Keywords: Peroxisome; DNA repair; Cell type specific gene regulation; Cell death; Superoxide dismutase; Reactive oxygen species

1. Introduction

Cells are continually exposed to a variety of DNA-damaging agents, which if left unrepaired, can cause mutations or cell death. To maintain genomic integrity, cells have a network of mechanisms to identify the specific types of DNA damage and mount the appropriate repair response. Damage repair pathways often overlap, as they share enzymatic components [1–3]. Moreover, it is becoming increasingly evident that they also share the signal transduction pathways which are used to activate them [4].

Oxidative stress which results from the generation of reactive oxygen species (ROS) is a significant source of cellular and DNA damage [5,6]. The major ROS are singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical. ROS exert their damaging effects on the cell by reacting with different macromolecules in the cell. However, the most important consequence of oxidative stress is thought to be DNA lesions which can be mutagenic and thereby lead to genomic instability [2,7,8].

ROS are produced endogenously as by-products of normal aerobic metabolism. The mitochondrial electron

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transport chain is the most prolific source of cellular ROS, producing the superoxide anion which is subsequently converted to hydrogen peroxide [9]. Alternatively, ROS can result from exposure to exogenous agents such as UV, γ-radiation and a wide variety of environmental oxidants. Some of the cellular toxicity to γ-radiation and ultraviolet light (UV) is mediated by the formation of ROS near the cell membrane [10–12]. It is estimated that 60–70% of DNA damage mediated by γ-radiation is due to the generation of hydroxyl radicals [13,14].

Although toxic in high concentrations, ROS also appear to play roles in signal transduction in a variety of distinct pathways. ROS activate signal transduction pathways which results in increased transcription of specific genes [15,16]. ROS have also been found to act as secondary messengers and to signal cellular fates such as cell proliferation or apoptosis. This indicates that cells must have mechanisms to regulate ROS levels which in turn control specific cellular responses [17–20]. The ROS levels in cells are modulated by a variety of antioxidant enzymes. Superoxide anions are converted to hydrogen peroxide by superoxide dismutase. Catalase, glutathione peroxidase and thioredoxin all function to convert hydrogen peroxide to water, preventing its decomposition to the hydroxyl radical [17]. Thus, antioxidants and antioxidant enzymes clearly play a role in cell signaling as well.

The cellular slime mold *Dictyostelium discoideum* has been widely used in studies of cell and developmental biology [21,22]. *Dictyostelium* cells are unusually resistant to UV-light, γ-radiation and DNA-damaging chemicals, but the basis for this is not well understood [23,24]. As such, *Dictyostelium* is particularly interesting for the study of DNA repair. Previously, we have cloned and characterized three nucleotide excision repair (NER) genes in *Dictyostelium*, *rep*B, D and E [25,26], which are the homologs of the human XPB and XPD DNA helicases and the damage recognition molecule UVDDB/XPE, respectively. In *Dictyostelium*, each of these genes shows a specific pattern of developmental regulation. *rep*D and E mRNA levels increase in early development, while *rep*B transcription is not altered. The mRNAs of the *rep*B and *rep*D genes are rapidly and robustly upregulated in response to UV, while the *rep*E mRNA level is simultaneously downregulated. In contrast, the expression of all three of these genes is upregulated in cells treated with the widely used antitumor agent cisplatin [23]. The different responses of the *rep* genes to UV, cisplatin and development indicate that they are regulated by multiple signal transduction pathways.

The UV-induced upregulation of NER genes in *Dictyostelium* has not been observed in other species, and thus may be at least partially responsible for the unique resistance of *Dictyostelium* cells to DNA-damaging agents. In this paper we show that *Dictyostelium* is also more resistant to oxidative stress than other organisms. This raised the possibility that *Dictyostelium* responds to ROS in a fashion analogous to the response to UV, by rapidly inducing antioxidant and/or oxidative repair enzymes. Furthermore, we wished to determine if ROS are involved in the rapid upregulation of the NER genes that we observed after exposure to UV-light.

In the present study, we report the identification and characterization of two catalase genes from *Dictyostelium*, *cat*A and *cat*B, and the Cu/Zn superoxide dismutase gene *sod*A. We found that the catalase genes are regulated independently both temporally and spatially during development. We have also investigated the response of these antioxidant genes, as well as genes involved in NER and base excision repair (BER), to oxidative damage and treatment with UV-light.

2. Materials and methods

2.1. DNA clones and sequence information

A full length cDNA clone for the catalase *cat*A gene (FC-AH16) and a partial clone for the catalase *cat*B gene (SSB660) were obtained from the cDNA sequencing project, Tsukuba, Japan [27] and were sequenced in our laboratory. The sequences were submitted to Genbank (accession number AF090443 for *cat*A and AF183177 for *cat*B). The remaining sequence for the *cat*B gene was later deduced from sequence data produced by the *Dictyostelium* sequencing group at the Sanger Center and from the partial sequence of a recently submitted full length *cat*B clone (SLK452) from the cDNA project.

A partial clone for the superoxide dismutase *sod*A gene (FC-AH19) was obtained from the cDNA project [27]. The partial sequence (missing the first two N-terminal amino acids of the *sod*A gene) was submitted to Genbank (accession number AF092899). The remaining sequence for the *sod*A gene was later obtained from Genbank (accession number AU037928) from a newly submitted clone (SSE463) from the cDNA project. The *sod*A gene is homologous to Cu/Zn superoxide dismutases.

The AP-endonuclease (*ape*A) clone was a gift from Dr. Reg Deering (Penn State, [28]).

2.2. DNA manipulations and preparation of probes

Restriction analysis, cloning and sequencing were performed according to standard methods [29]. Northern and Southern analyses were described in detail elsewhere [30] and were quantitated with a Fuji phosphorimager. The rRNA in the RNA gels was stained with acridine orange to ensure equal loading and transfer to the hybridization membranes. The probes for *cat*A (1189 bp), *cat*B (480 bp), *rep*B (462 bp) and *rep*D (814 bp) were generated by PCR, using plasmid DNA as templates [30]. The probes for *sod*A (540 bp) and *ape*A (500 bp) were excised as *Not*I–*Sal*I or *Hin*dIII fragments from their respective plasmids.

2.3. RT-PCR

RT-PCR was performed using a SuperScript preamplification system (Life Technologies, Grand Island, NY, USA), following the manufacturer's instructions. DNaseI treated samples were reverse and amplified transcribed using *cat* gene specific primers. *cat*A and *cat*B cDNA plasmid preparations were used as templates to confirm the specificity of each set of primers for its cognate gene.

2.4. Strains and conditions for growth and development

Strain Ax4 was grown in HL-5 axenic medium [31]. Cells were harvested for development, or passed when they reached a density of $2–3\times10^6$ cells/ml. Strains NC4, WS380B and X9 were grown on SM agar plates in association with *Klebsiella aerogenes* until mid-log phase (approximately 2×10^8 cells/100 mm plate [32]). NC4 is the standard bacterially grown wild type strain and WS380B is another wild isolate that develops more rapidly [33]. Mutant strain X9 carries chromosome II from strain ts12, to which a mutation affecting catalase activity had been previously mapped by parasexual genetics [34]. The chromosome is marked by the *whi*A1 and *tsg*D12 alleles [35]. All cultures were routinely restarted monthly from stocks.

For development, cells were harvested, washed in cold LPS buffer (20 mM KCl, 2.5 mM $MgCl_2$, 40 mM K-PO_4, pH 6.5 containing 0.5 mg/ml streptomycin sulfate) until free of medium or residual bacteria and resuspended in LPS at a concentration of 2×10^8 cells/ml. 1×10^8 cells were placed on LPS saturated black filter paper disks for development [32,36]. Morphogenesis is synchronous under these conditions.

2.5. Viability assays

An aliquot of cells was sampled after thoroughly mixing the culture and was serially diluted in SS/EDTA (10 mM NaCl, 10 mM KCl, 2.7 mM $CaCl_2$, 5 mM EDTA). Dilution samples were plated on SM agar in association with *K. aerogenes*. A cell capable of sustained growth gives rise to a plaque on the bacterial lawn.

We have also developed a technique to determine the percentage of dead cells in the culture, based on the ability of live cells to exclude the dye phloxine B. The addition of dye to a final concentration of 0.01% to a sample stains dead cells bright red in less than a minute and they can be conveniently scored with a hemocytometer.

2.6. Hydrogen peroxide treatment

Logarithmically growing Ax4 cells in HL-5 medium ($1–2\times10^6$ cells/ml) were treated with the indicated concentrations of hydrogen peroxide. Samples were taken at indicated time points and were analyzed for viability, for mRNA expression and for enzyme activity levels. Prior to each experiment, the concentration of the hydrogen peroxide solution was checked by reading the A_{240} of a 1/800 dilution of the 30% stock in H_2O. The extinction coefficient of hydrogen peroxide at 240 nm is $43.6A_{240}$ M^{-1} cm^{-1}.

2.7. UV treatment

Wild type NC4 or catalase mutant X9 cells were grown on SM agar plates in association with *K. aerogenes*. Cells were harvested, washed with LPS and resuspended in LPS containing 5 mM EDTA to a density of 2×10^6 cells/ml. UV treatment was performed as described previously [23]. Briefly, 10 ml of cell suspension were shaken in sterile Pyrex Petri dishes and irradiated with 254 nm UV-light (germicidal lamp ITT G15T8) at varying fluences. The incident fluence was measured by using a Black Ray meter (UVP, San Gabriel, CA). UV fluences were corrected for the path length and the transmission of the cells using the method described by Morowitz [37]. Following irradiation, the cells were allowed to recover for 40 min and then sampled to determine viability. The remaining cells were harvested and used for Northern analyses and enzyme activity assays.

2.8. Cell type separation

Prestalk and prespore cell populations from *Dictyostelium* preculminants were separated based on the method developed by Ratner and Borth [38]. NC4 amoebae were allowed to develop on LPS-agar plates, harvested late in development into cold LPS buffer and pelleted. The culminants were disaggregated by incubation in 4 volumes of disaggregation medium (50 mM Tris–HCl, pH 7.0, 10 mM EDTA, 25 mM 2,3-dimercaptopropanol, 0.1% pronase) [39] for 5 min at room temperature followed by trituration (5 times) with a 16 gauge 1.5 inch needle attached to a syringe. The suspension was then brought up to 50 ml with LPS and passed through a 70 μm nylon mesh. The cells were pelleted, washed twice with LPS containing 5 mM EDTA and resuspended to a density of 4×10^8/ml in 20 mM EDTA, 5 mM MES, pH 7.0.

Twelve ml 55% Percoll gradients (in 20 mM EDTA, 5 mM MES, pH 7.0) were preformed by centrifugation in a Beckman JA-20 rotor for 40 min at $26\,890\times g$ at 4°C. The gradients were calibrated using density marker beads (Sigma, St. Louis, MO, USA). Two hundred and fifty μl ($\sim1\times10^8$ cells) of the cell suspension were layered on top of each gradient and centrifuged in a Beckman JS-13.1 rotor for 5 min at $23\,750\times g$. Prestalk and prespore cell populations were observed as two separate bands. To achieve greater purity, the prespore and prestalk bands were collected separately, pooled and rebanded on a second 55% Percoll gradient. The harvested cells were diluted 5–10 times with LPS and pelleted to remove the Percoll.

Purity of the cell populations was assessed by Western analyses using cell type specific antibodies.

2.9. Subcellular fractionation

WS380B cells were allowed to develop on nitrocellulose filters to the preculminant stage. Aggregates were harvested and disaggregated as described above. The cells were then washed twice with LPS and resuspended in 3 volumes of cold homogenizing medium (50 mM Tris, pH 7.6, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1× protease inhibitor cocktail (100× = 20 mM AEBSF, 100 μg/ml pepstatin A, 10 μg/ml leupeptin)). The cells were broken with 30 strokes of a Dounce homogenizer. Unbroken cells, debris and nuclei were removed by centrifugation at 1000×*g* at 4°C in a Beckman JS-13.1 rotor [40]. The supernatant was then fractionated by sequential centrifugations at 9000 and 20 000×*g*. Samples of the homogenate, pellets and supernatants were analyzed for their associated catalase activities using the native gel catalase assay. Urate oxidase activity which is a marker for peroxisomes was assayed as described [41].

2.10. Spectrophotometric catalase assay

Catalase activity was determined using a modification of the method of Aebi [42]. 1×10^7 cells were lysed in 100 μl lysis buffer (10 mM K-PO_4, pH 7.0, 0.1% Triton X-100, 1× protease inhibitor cocktail) for 3 min, on ice and centrifuged for 3 min at 4°C. 1.25–5 μl of the supernatant were placed in a quartz cuvette followed by 1 ml of 10 mM H_2O_2 solution in 50 mM K-PO_4, pH 7.0. Hydrogen peroxide degradation was monitored by recording the absorbance at 240 nm every 10 s for 90 s. Specific activity of catalase was expressed as μmol of H_2O_2 degraded per minute per mg protein. Prior to doing each assay, the concentration of the stock H_2O_2 solution was verified as described earlier. Specificity of this assay for catalases in *Dictyostelium* lysates was confirmed by using 3-amino-1,2,4-triazole [43] and mercaptosuccinate [44], which are direct enzyme inhibitors of catalase and glutathione peroxidase, respectively. Protein concentrations were determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA).

Table 1
Comparison of *Dictyostelium* catalases with catalases from different organisms

Organism (accession number)	Size (amino acids)	p*I*[a]	Percent similarity with *Dictyostelium* CatA[b]	Percent similarity with *Dictyostelium* CatB[b]
Dictyostelium discoideum (AF090443) CatA	497	8.49		44.1
D. discoideum (AF183177) CatB	693	6.67	44.1	
Animal catalases				
Bos taurus (P00432) CatA	506	6.70	56.5	40.2
Caenorhabditis elegans (X82175) Cat	500	7.84	57.4	40.5
Drosophila melanogaster (X52286) CatA	506	6.32	57.1	41.2
Homo sapiens (X04076) CatA	527	7.22	57.2	40.3
Mus musculus (M62897) CatA	527	7.82	56.2	40.6
Rattus norvegicus (M11670) CatA	527	7.36	56.4	40.6
Plant catalases				
Arabidopsis thaliana (Q96528) Cat1	492	7.26	44.7	43.0
A. thaliana (P25819) Cat2	492	6.93	45.3	42.7
A. thaliana (AAC49807) Cat3	492	5.90	46.9	44.4
Zea mays (X12538) Cat1	492	7.25	47.1	45.6
Z. mays (X54819) Cat2	491	6.97	45.5	42.4
Z. mays (L05934) Cat3	496	6.77	43.9	41.2
Fungal catalases				
♦ *Emericella nidulans* (U37803) CatA	744	6.28	40.0	48.5
E. nidulans (U80672) Cat B	721	4.81	40.9	41.9
Saccharomyces cerevisiae (X13028) CatA	515	7.31	50.5	36.1
S. cerevisiae (X04625) CatT	562	6.44	43.5	34.7
Bacterial catalases				
♦ *Bacillus firmus* (M74194) KatA	448	5.73	46.4	63.6
Bacillus subtilis (M80796) KatA	483	6.34	60.7	45.3
▲ *B. subtilis* (X85182) KatE	529	6.44	45.5	61.2
♦ *B. subtilis* (BAA11740) KatX	547	5.52	44.4	38.2
▲ *Escherichia coli* (M55161) KatE (HPII)	753	5.67	44.9	53.3
E. coli (M21516) KatG (HPI)	726	5.06	20.1	20.7
Helicobacter pylori (U67458) Kat	505	8.68	53.7	42.6
Pseudomonas putida (U63511) CatA	479	6.65	58.3	43.0
▲ *P. putida* (U82622) CatC	711	6.45	45.4	58.7

♦ spore specific; ▲ stationary phase specific

[a] p*I*s were determined using the Protean program (DNASTAR).

[b] Percent similarity was determined using the Jotun-Hein algorithm in MegAlign (DNASTAR).

2.11. Native gel catalase assay

In order to distinguish the activities of each specific catalase, the proteins were separated on a native polyacrylamide gel and the catalase activity was assayed as described [45]. *Dictyostelium* cells were lysed in native sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 1% NP-40, 0.05% bromphenol blue, 1× protease inhibitors) for 3 min on ice and centrifuged for 3 min at 4°C. Cell lysates (5×10^6–1×10^7 cell equivalents/lane) were electrophoresed on a native polyacrylamide gel (62.5 mM Tris, pH 6.8–4% stacking gel/375 mM Tris, pH 8.8–7.5% resolving gel) at 120 V, for 5 h at 4°C in Tris–glycine, pH 8.3 running buffer. After electrophoresis, the gel was incubated in 50 mM K-PO_4, pH 7.0 for 15 min and then in 0.03% H_2O_2 solution in H_2O (1/1000 dilution of 30% stock) for 10 min. The gel was rinsed twice with H_2O and then incubated in a mixture (1:1) of freshly prepared 2% $K_3Fe(CN)_6$ and 2% $FeCl_3$. The gel became greenish-blue while zones of catalase activity were yellow.

In the gel assay, CatA activity was always observed at the boundary of the stacking and resolving gels even if the gel was run overnight. This is in contrast to CatB activity which was observed to enter the resolving gel. The localization of CatA activity to the upper part of the gel is attributed to its high p*I* (=8.49) relative to the catalases from other organisms (see Table 1). Using a gel at lower pH (pH 6.0–6.8) and electrophoresing the proteins towards the cathode were not successful because the detection reagent was incompatible with the low pH of the gel. Using a gel at higher pH (pH 10.6) was not successful either because the enzyme activity was lost after several hours of electrophoresis at this pH.

3. Results

3.1. Characterization of Dictyostelium catalase genes

We have identified and characterized two distinct, but highly related catalase genes in *D. discoideum*, which were named *cat*A and *cat*B. Southern analyses of these genes demonstrate that they are unique (Fig. 1). The *cat*A gene is 1491 bp long and contains an 82 bp intron at position

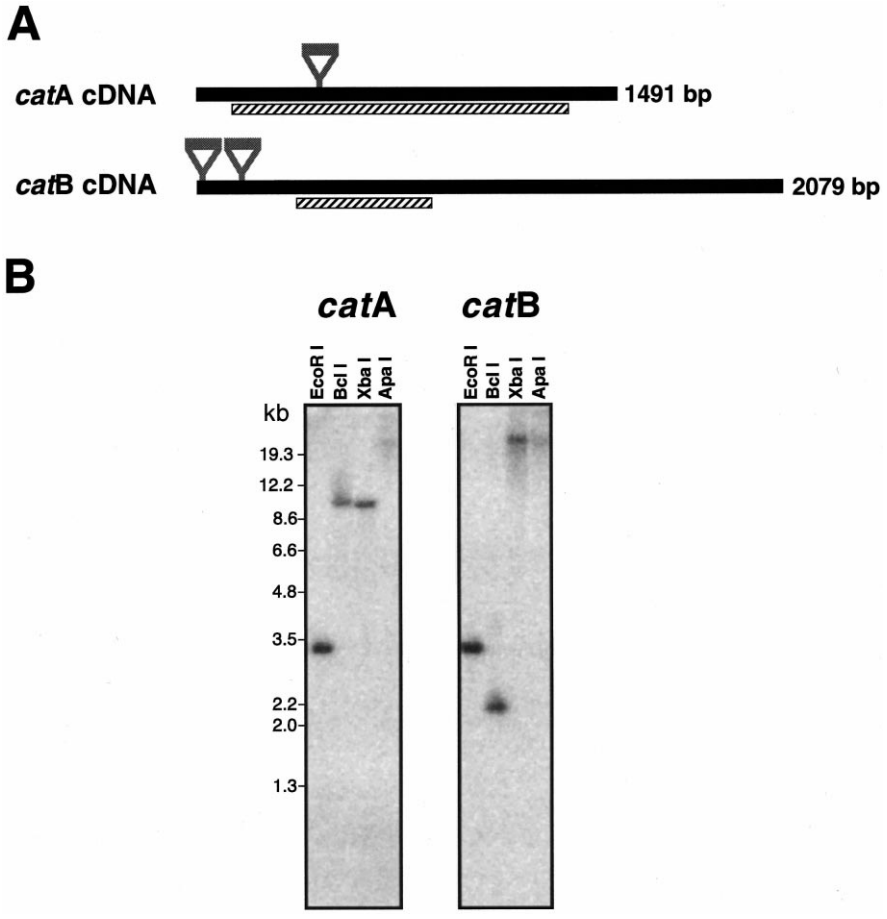


Fig. 1. *cat*A and *cat*B are two distinct loci in the *Dictyostelium* genome. (A) Schematic presentation of the *cat*A and *cat*B genes showing their relative sizes and intron positions. (B) Restriction analysis of the genomic regions of *cat*A and *cat*B. Twenty five μg of genomic DNA were digested with the indicated restriction enzymes, separated on a 0.8% agarose gel in TEA buffer and blotted onto a nylon membrane. The membranes were probed at 42°C and 50% formamide with *cat*A and *cat*B probes, respectively, indicated as hatched rectangles in (A).

444 (verified by comparing PCR products from genomic DNA and cDNA [46]). It encodes a putative 497 amino acid long polypeptide, with an expected molecular mass of 55.7 kDa and an unusually high p*I* of 8.49 relative to other catalases (see Table 1).

The *cat*B gene was initially identified in a REMI suppressor screen of a double mutant lacking both the *car*1 and *car*3 cAMP receptors (unpublished). *Dictyostelium* strains mutated in these *car* genes are unable to respond to cAMP signals and, therefore, are unable to aggregate and develop [47]. One of the aggregation competent suppressed strains was found to have an insertion in the *cat*B gene. The mechanism of this suppression is currently under investigation. The remaining sequence for *cat*B was obtained from a clone acquired from the cDNA project and from the genomic database. The *cat*B gene is 2079 bp long and contains two introns, a 75 bp intron at position 16 and a 119 bp intron at position 163 (verified by

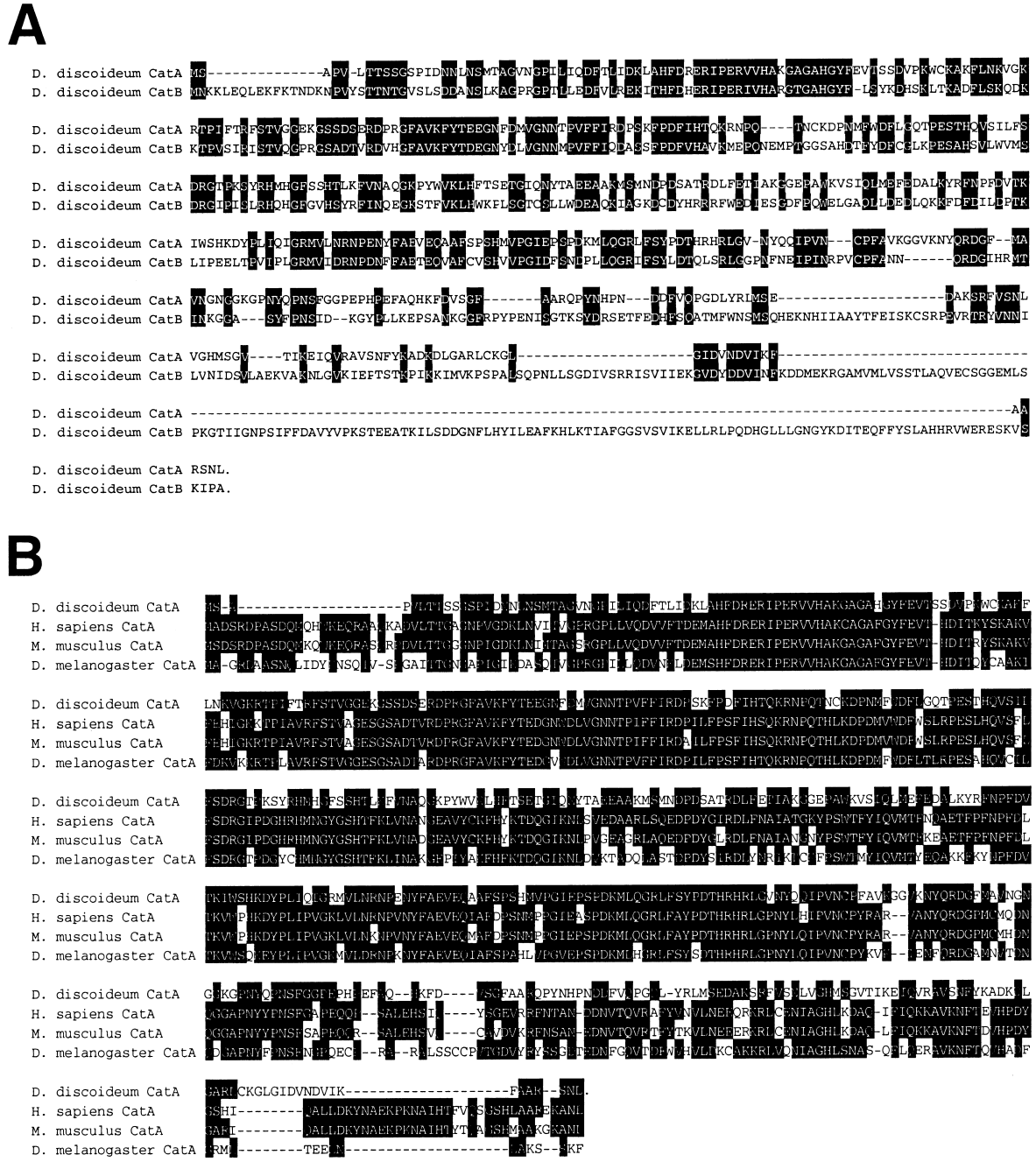


Fig. 2. Comparison of catalase protein sequences. (A) Similarity of the CatA and CatB proteins. Similarity of CatA (B) and CatB (C) proteins with representative catalases from other organisms. Alignments were performed using the Jotun Hein method with the PAM 250 residue weight table (MegAlign Program, DNASTAR), allowing 1 unit of distance.

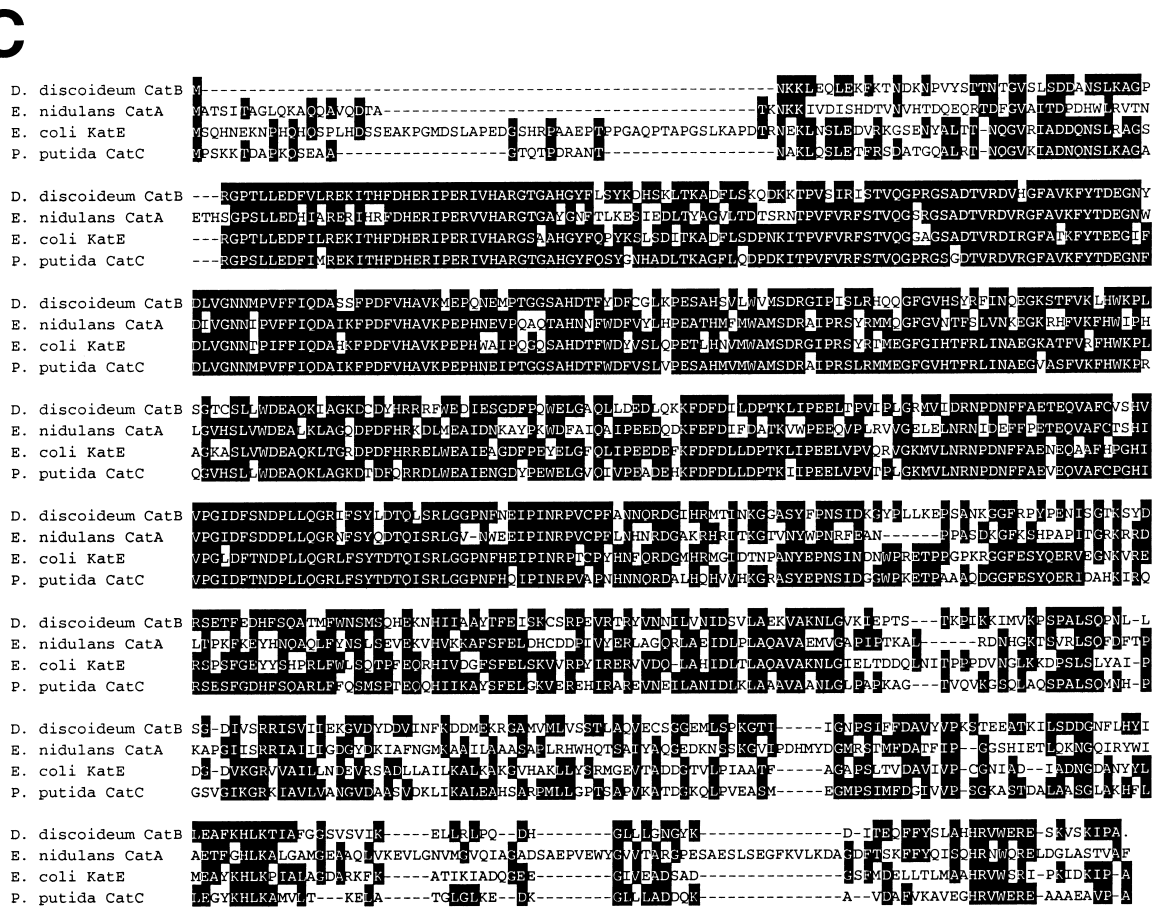


Fig. 2 (*continued*).

comparing the cDNA and genomic DNA sequences obtained from the Tsukuba cDNA project and the Sanger Center genome sequencing databases, respectively). *cat*B encodes a 693 amino acid long protein, with an expected molecular mass of 78.0 kDa and a p*I* of 6.67.

Sequence analysis revealed that both catalases are highly related to each other having a 44.1% overall similarity, although the similarity is lower in the region representing the 97 C-terminal amino acids of CatA (Fig. 2A). Both catalase gene products show significant similarity to catalases from other organisms, as is shown in Fig. 2B and C and Table 1. Generally, the highest similarity among all catalases is observed in the first two thirds of the polypeptide, while the C-termini are less conserved among species. CatA shows higher similarity to catalases from organisms such as human, mouse and fly which all have only one catalase gene. CatB is more similar to the higher molecular weight catalases (Table 1). Furthermore, in organisms which have more than one catalase, CatB is often more similar to the sporulation specific or stationary phase inducible catalases, such as the *Emericella nidulans* CatA [48], the *Pseudomonas putida* CatC [49], the *Bacillus subtilis* KatE [50] and the *Escherichia coli* KatE [51] (denoted by diamonds and triangles in Table 1).

3.2. Developmental regulation of the cat genes

The levels of the *cat*A and *cat*B gene products were determined in growing cells and at every hour during the 24 h time course of development. Northern analysis showed that there is a fairly constant level of *cat*A mRNA at all stages of growth and development with a slight (less than two-fold) increase in *cat*A mRNA during the first 5 h of development (Fig. 3A). In contrast, *cat*B shows dramatic developmental regulation. There is no *cat*B mRNA in growing cells and for the first 10–11 h of development. It first appears when the aggregates form apical tips, accumulates rapidly during the time of terminal cell differentiation and peaks at about 21 h (Fig. 3A). RT-PCR experiments confirmed that there is no detectable *cat*B mRNA in growing cells or in cells from the early stages of development (Fig. 3B). The *sod*A gene is expressed at a constant level throughout growth and development (data not shown).

The corresponding enzyme activity was measured in two different ways: by a spectrophotometric assay, which measures total catalase activity in the cells and by native gels, where the activity of each catalase species can be distinguished. As is shown in Fig. 3C, where total catalase

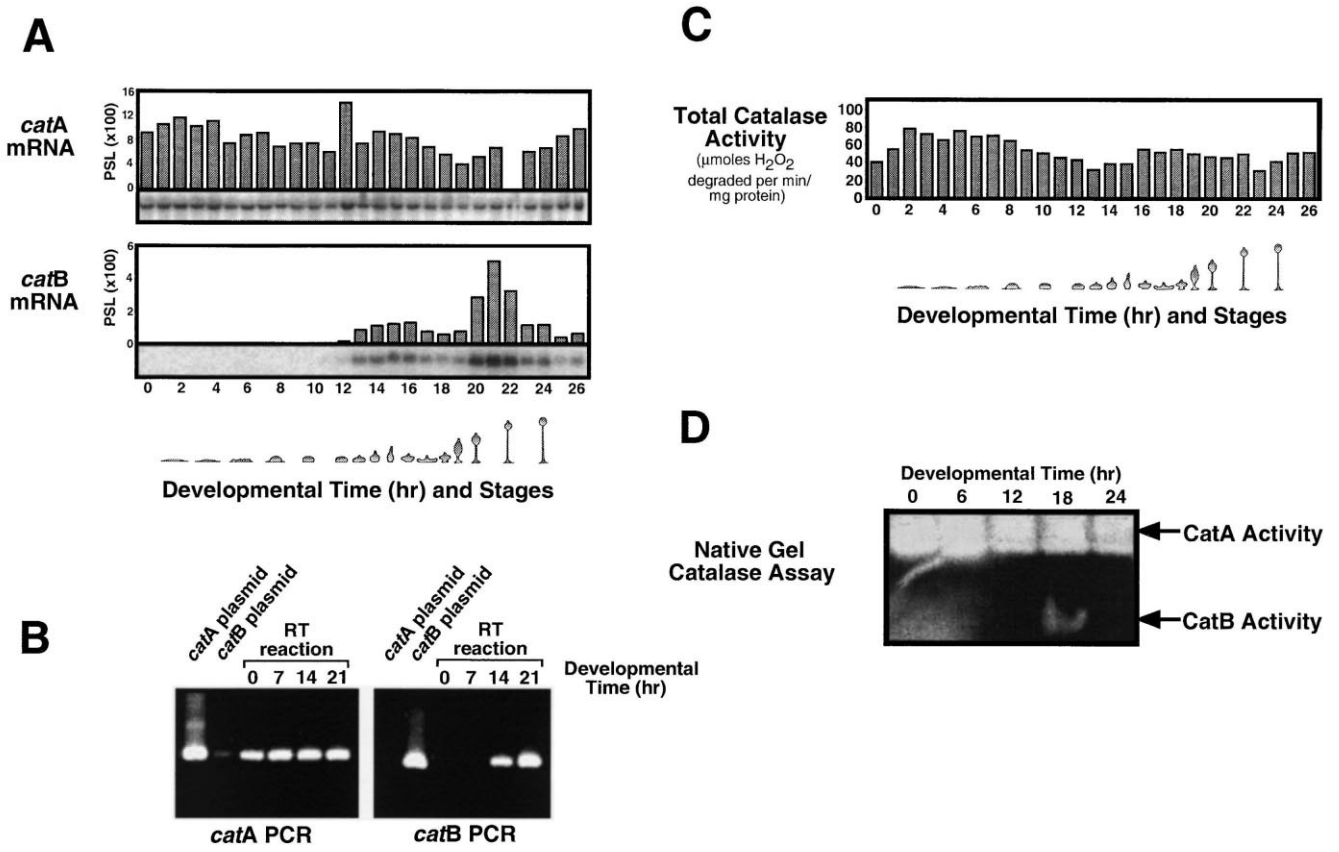


Fig. 3. Developmental regulation of *cat*A and *cat*B expression. Ax4 cells were allowed to develop synchronously on filters. Samples were harvested hourly during development as described in Section 2. (A) Total RNA samples were prepared from each time point, separated on a 1.0% agarose-formamide gel and blotted onto a nitrocellulose membrane. Membranes were hybridized with either *cat*A or *cat*B probes and the expression level was quantitated with a Fuji Phosphorimager. Units are photostimulated luminescence (PSL). (B) RT-PCR analysis of *cat*A and *cat*B expression at representative developmental time points. *cat*A and *cat*B plasmids were used as controls. (C) Cell lysates from each time point were assayed for total catalase activity. (D) Native gel catalase assay of representative time points during development.

activity is measured, the specific activity is quite constant throughout growth and development. It is interesting to note that there is no burst of catalase activity in late development to match the abrupt onset of *cat*B mRNA accumulation. This is also reflected by the gel assays, in which we can see the abrupt appearance of CatB enzyme activity and observe that it is low, compared to the activity of CatA (Fig. 3D).

3.3. Cell type specificity of catalases

The induction of *cat*B expression late in development raised the possibility that CatB is a sporulation specific enzyme. To test this we separated prespore and prestalk cells on a Percoll density gradient [38]. We achieved highly enriched populations of prespore and prestalk cells, as judged by Western analyses using cell type specific antibodies (Fig. 4A). A native gel catalase activity assay revealed that the CatB activity was associated with the prespore cells. Unexpectedly, CatA activity was associated only with prestalk cells (Fig. 4B). Therefore, the regulation of the catalase genes is on both the temporal and spatial levels. Based on these results, the CatB enzyme is expected to be in the spores which are resistant to detergent extraction. This may explain why there is no CatB activity observed in the last developmental timepoint in Fig. 3D.

3.4. Subcellular localization

Both catalases have putative peroxisome targeting sequences (PTS). In the case of CatA, the four most C-terminal amino acids are RSNL, similar to the C-terminal KANL, which has been shown to be the targeting sequence of human catalase [52]. CatB has two adjacent putative peroxisome targeting signals at the C-terminus, SKV/SKI, which are similar to the PTS1 of many organisms [53]. In addition, CatB, but not CatA, has a lysine rich region at the N-terminus which is consistent with putative mitochondrial targeting signals [54]. To test whether both *Dictyostelium* catalases localize to peroxisomes in their respective cell types, wild type WS380B cells were harvested in late development and were subjected to subcellular fractionation using sequential centrifugations. The results show that CatA and CatB activities have different cellular localization (Fig. 5). We show here that CatA activity sediments between 9000 and 20 000 $\times g$ and

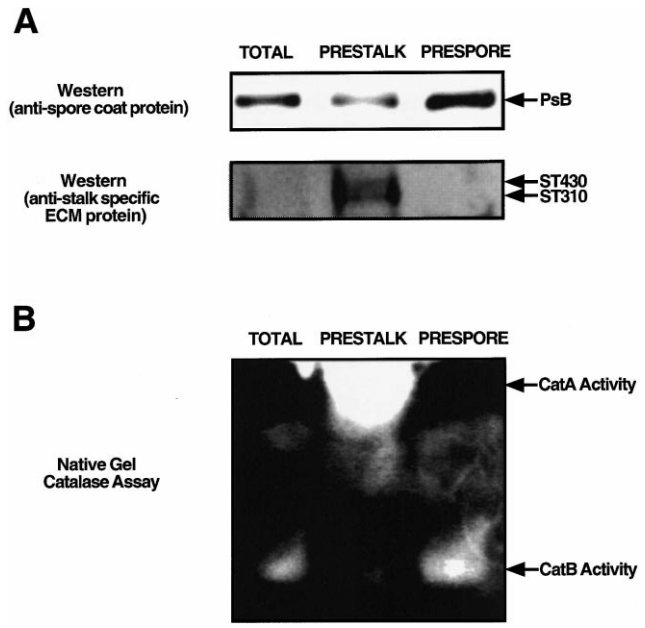


Fig. 4. Cell type specificity of CatA and CatB enzyme activities. Enriched populations of prespore and prestalk cells were obtained as described in Section 2. (A) The purity of the cell populations was determined by Western analyses, using MUD102 and Jab2 antibodies, which recognize the spore coat protein PsB [86,87] and the stalk-specific extracellular matrix (ECM) proteins ST310 and ST430 [88], respectively. (B) Specific catalase activities associated with the two cell populations were determined using the native gel catalase assay. Equal protein amounts (40 μg per lane) of total, prestalk or prespore cell samples were loaded on the native polyacrylamide gel.

co-fractionates with urate oxidase (Fig. 5, bottom panel), which is a marker for peroxisomes [41]. On the other hand, the CatB activity did not sediment and remained in the 20 000×*g* supernatant. Thus, CatB is not peroxisomal despite having putative peroxisome targeting sequences. These experiments show that the catalase activity previously demonstrated in peroxisomes of growing cells is exclusively CatA [55,56].

3.5. *catA* mutant

Derivatives of strain ts12, including strain X9, have greatly reduced levels of catalase activity in growing cells (see Section 2 for strain lineage; [34]). We have now further characterized this mutation. Since we established that *cat*B is expressed late in development, it became clear that the mutation affects *cat*A. Fig. 6A shows that strain X9, which carries the mutation, expresses less than 1% of the *cat*A mRNA relative to the wild type, with a correspondingly low level of enzyme activity (Fig. 6B). Both mutant and wild type cells express *cat*B mRNA, and the corresponding enzyme activity, to the same extent and at the same time in development (Fig. 6A and B), showing that the two genes are regulated independently. These results suggest that the mutation in *cat*A is regulatory in nature and sequencing of the mutant *cat*A gene confirmed that there were no changes in the coding sequence. Moreover, it is clear that CatB is not abnormally expressed in the mutant to compensate for the lack of CatA.

We determined that the *cat*A mutant X9 is 160-fold more sensitive to H_2O_2 compared to the wild type NC4. The 0.5 mM H_2O_2 treatment resulted in 0.58% survival in the mutant relative to 80.7% in the wild type (Fig. 6C).

3.6. Response of *Dictyostelium* catalase genes to stress

As stated above, ROS can be formed in the cells as a result of normal metabolism or as a result of exposure to oxidative agents. We wished to determine whether *Dic-*

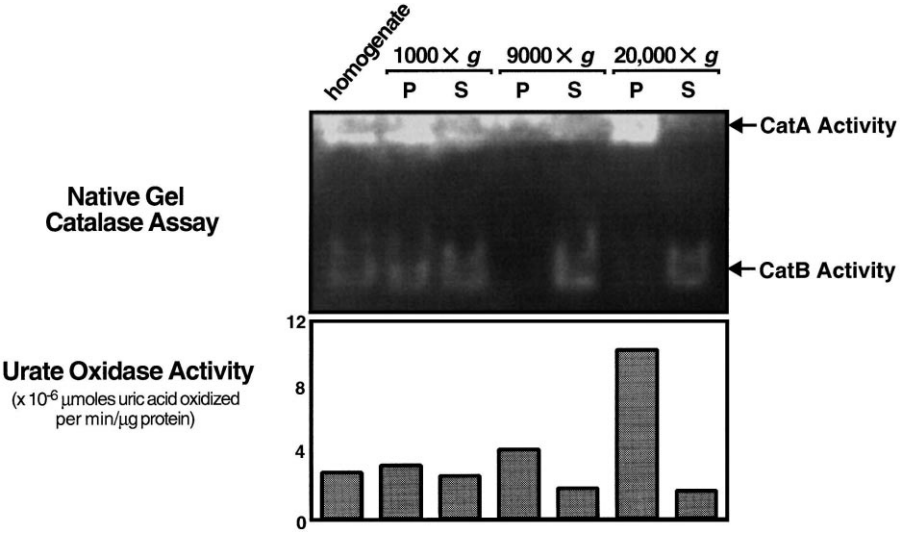


Fig. 5. Subcellular fractionation of catalase activities. Wild type WS380B cells were harvested in late development, disaggregated and broken with a Dounce homogenizer. The homogenate was subjected to sequential centrifugations. (A) Equal protein amounts of the fractions (60 μg per lane) were loaded on a native polyacrylamide gel. Specific catalase activities associated with the fractions were determined using the native gel catalase assay. (B) The fractions were assayed for urate oxidase activity which is a peroxisomal marker.

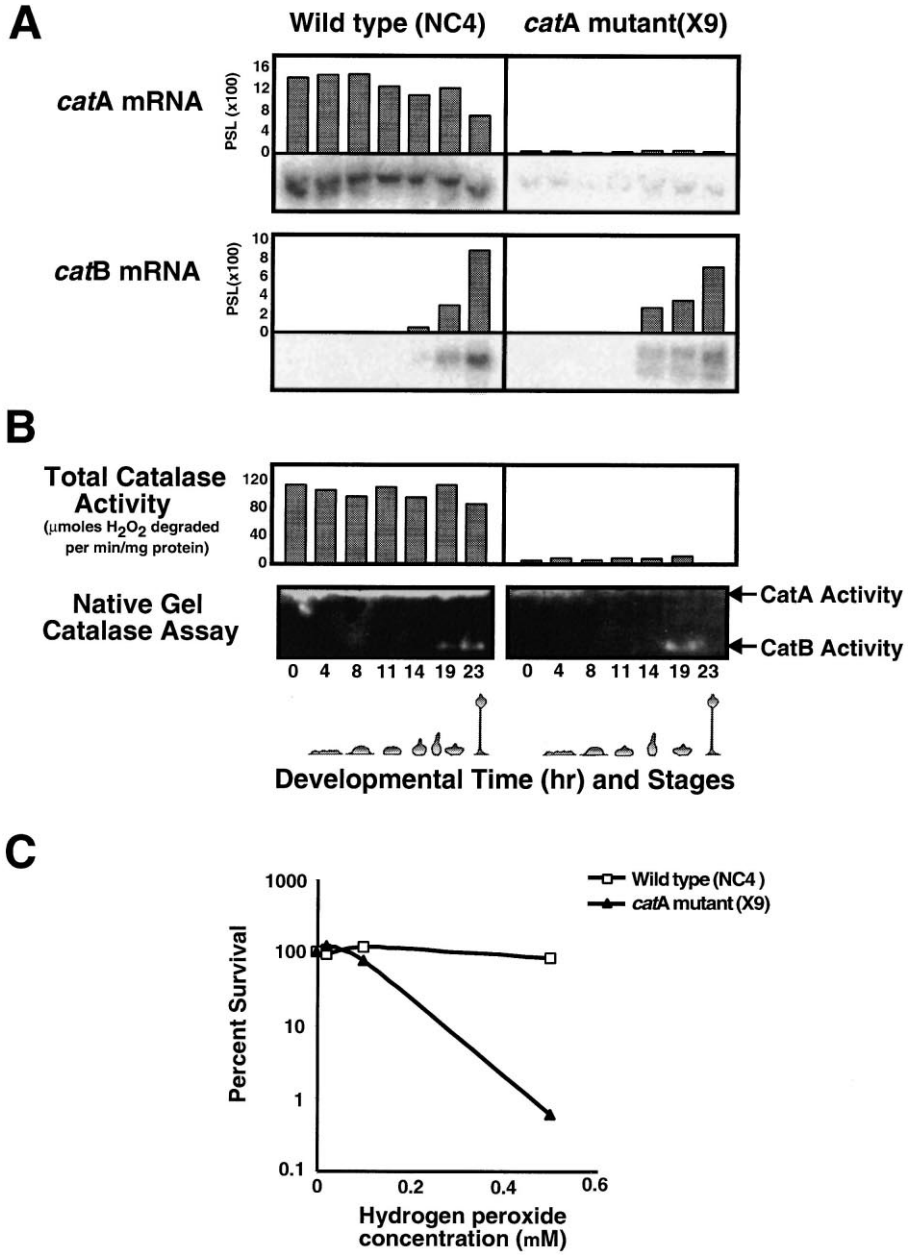
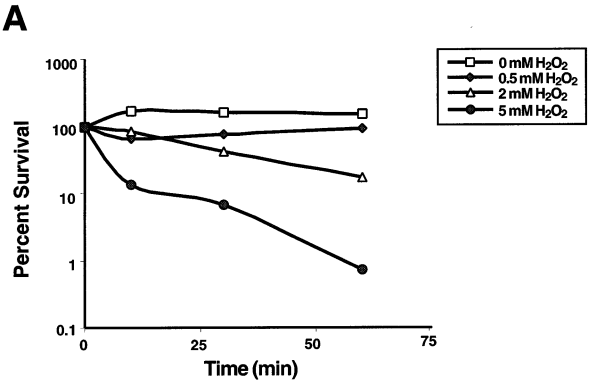


Fig. 6. *cat*A and *cat*B gene expression and H_2O_2 sensitivity in wild type and catalase deficient mutant. NC4 (wild type) and X9 (*cat*A mutant) cells were allowed to develop synchronously on filters. Samples were harvested at the indicated time points and analyzed for (A) *cat*A and *cat*B mRNA levels and for (B) enzyme activities. (C) X9 and NC4 cells were resuspended in LPS buffer, treated with varying concentrations of H_2O_2 and analyzed for cell viability.

tyostelium cells respond to these conditions by modulating the levels of expression of their catalases.

3.6.1. Growth conditions

The level of catalase activity in non-axenic, bacterially grown *Dictyostelium* NC4 cells was reported to be relatively higher than that in other organisms [34]. To test whether this was possibly in response to continuous production of ROS by the bacterial food source, we determined the levels of catalase in Ax4 cells that were grown axenically in HL-5 medium, in Ax4 cells that were grown in buffered suspensions of live or dead (autoclaved) bacteria and in Ax4 or NC4 cells grown on bacterial lawns. We did not detect a marked difference in the catalase levels in response to the different growth conditions in liquid, whether they were grown axenically, or in the presence of live or dead bacteria. Ax4 cells grown on a bacterial lawn exhibited a marginal (20–30%) increase in the level of catalase compared to cells that were grown in the various liquid cultures. However, NC4 cells grown on a similar bacterial lawn, had up to four-fold higher catalase activity than Ax4 grown in the same conditions and, thus, we attribute this difference to strain variations (data not shown).



B

H_2O_2 concentration (mM)	0	0.5	5
Percent phloxine B stained cells after 24 hours	0	2.2	93.1

Fig. 7. Survival of *Dictyostelium* cells after treatment with H_2O_2. Logarithmically growing Ax4 cells in HL-5 medium ($1–2\times10^6$ cells/ml) were treated with different concentrations of H_2O_2. (A) Samples were taken at indicated time points and were analyzed for viability by plating on bacterial lawn. (B) Samples were assayed by the cell's ability to exclude phloxine B dye. The stained cells represent the dead cells in the population.

We also examined the levels of catalase mRNA and enzyme activity after cells had reached stationary phase. Cultures were sampled daily, starting in the early log phase, and continuing three days into stationary phase. The specific activity of CatA enzyme remained constant during the logarithmic growth phase and during the stationary phase of the culture. It is interesting to note that even though CatB has a high homology with the stationary phase inducible catalases of other organisms, it is not induced during stationary phase in *Dictyostelium*.

3.6.2. Response to H_2O_2 treatment

We then wished to test if the catalase genes respond to H_2O_2 as an exogenous source of oxidative stress. To enable us to do that, we first needed to establish a sublethal dose of H_2O_2 that would allow us to follow induction of response genes.

We determined the sensitivity of growing cells to H_2O_2 two ways: cells treated with H_2O_2 were clonally plated for survival on agar plates with a lawn of *K. aerogenes*. Alternatively, the cells were tested for their viability by their ability to exclude the vital dye phloxine B. In a preliminary experiment, we treated growing cells with different concentrations of H_2O_2 and observed that the extent of killing by H_2O_2 was dose-dependent (Fig. 7A). The 5 mM H_2O_2 treatment resulted in a dramatic reduction in viability (13.5% survival) after only 10 min. The 0.5 mM H_2O_2 treatment appeared to be sublethal, resulting in 95% viability after 60 min. The viability does not change appreciably even after 4 h of treatment (see Fig. 8).

When we evaluated cell survival at 0.5 and 5 mM H_2O_2 by phloxine B staining, we found that the extent of killing was consistent with the results based on plating. The cells do not die immediately after 5 mM H_2O_2 treatment as they do not take up the dye even several hours after treatment. However, after 24 h, 93.1% of the cells stained with the dye (Fig. 7B). In contrast, only 2.2% of the cells treated with 0.5 mM H_2O_2 stained red although the cells did not divide during that 24 h. Interestingly, after 24 h, the surviving cells began dividing again at the same rate as untreated control cells. This indicates that they were able to repair the damage and that the arrest in the cell cycle was transient and reversible. Cell cycle arrest during oxidative stress has also been demonstrated in yeast and mammalian cells [57–60].

Using 0.5 mM H_2O_2 as the sublethal dose for *Dictyostelium* cells, we analyzed the response of the *cat* genes to oxidative stress. Growing cultures were treated with the indicated amounts of H_2O_2 and sampled at indicated intervals for analysis of catalase gene expression, as well as for catalase enzyme activity (Fig. 8). There was no change in total catalase enzyme activity or in the steady-state levels of the *cat*A, *cat*B and *sod*A mRNAs or in the mRNA of the previously characterized BER gene *ape*A, following exposure to H_2O_2. The levels of mRNA from the NER genes *rep*B and *rep*D, which have been shown to

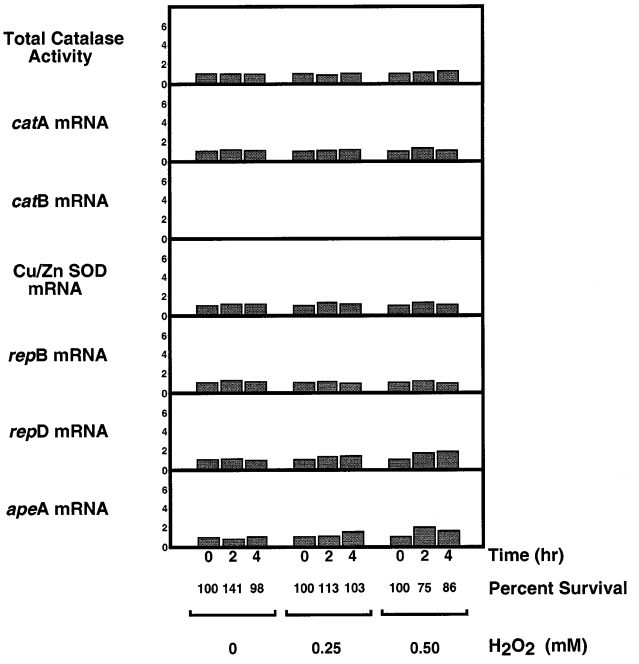


Fig. 8. Response of antioxidant enzyme genes and the BER and NER genes to sublethal levels of H_2O_2. Vegetative Ax4 cells were treated with 0, 0.25 or 0.5 mM of H_2O_2 for 0, 2 or 4 h. Total catalase activity was determined as described in Section 2. Total RNA (7.2 μg per lane) was analyzed for the level of expression of different antioxidant enzyme and repair genes, using gene-specific probes. Enzyme activity and message levels are depicted as fold over the level in untreated cells. Percent survival is indicated at the bottom of the figure.

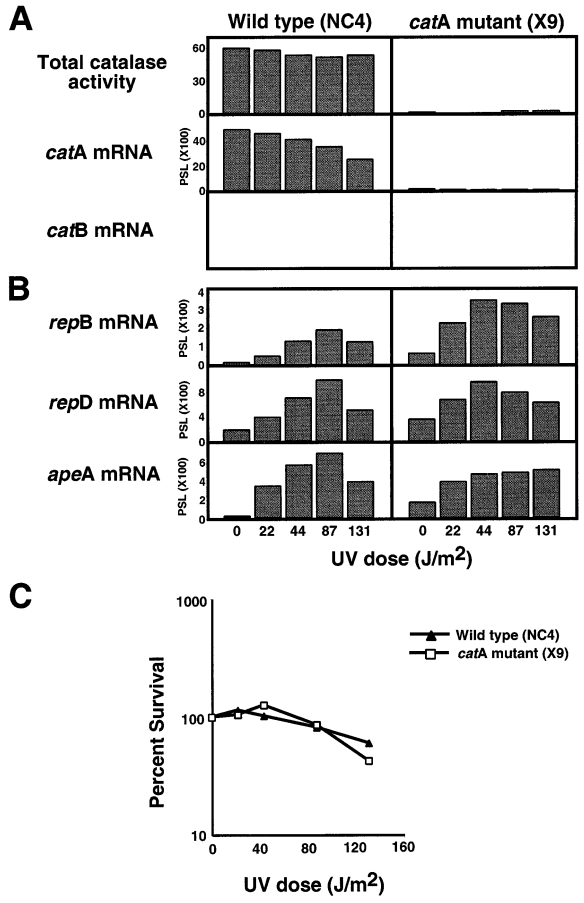


Fig. 9. UV response of antioxidant genes, NER genes and the BER gene in a wild type and catalase deficient background. Vegetative cells of wild type (NC4) and *cat*A mutant (X9) strains were harvested from clearing bacterial plates. Cells were washed and resuspended in LPS buffer at a density of 2×10^6 and irradiated at indicated fluences. (A) Total catalase activity was determined as previously described. Total RNA (7.2 μg per lane) was separated on a 1.0% agarose gel and blotted onto nitrocellulose. Membranes were probed with gene-specific probes for (A) the antioxidant genes *cat*A and *cat*B or for (B) the NER *rep*B and *rep*D genes and BER *ape*A gene. (C) Survival curves of the wild type and *cat*A mutant strains after UV treatment. Serial dilutions of cells were plated on a lawn of *K. aerogenes*. Percent survival was determined by comparing colony formation relative to unirradiated cells.

respond to UV and cisplatin, were also unaffected by exposure to H_2O_2, re-affirming the specificity of the induction of *rep* genes by UV and cisplatin.

3.6.3. Response to UV

Because UV has been observed to cause oxidative stress and produce oxidative DNA damage, we wanted to determine whether the antioxidant enzymes were induced in cells exposed to UV. We also wanted to determine whether catalase activity can modulate the cellular responses to UV. To this end we analyzed the UV induced gene expression and cell survival of the wild type and *cat*A mutant cells treated with UV.

The catalase mRNA levels or enzyme activities were not induced by exposure to UV-light (Fig. 9A). We also showed that even though the *cat*A mutant cells have significantly reduced levels of antioxidant enzyme, the NER and BER genes were upregulated by UV to the same extent as wild type cells (Fig. 9B). In addition, the wild type and *cat*A mutant exhibited similar survival curves in response to the UV treatment (Fig. 9C).

4. Discussion

Catalases are ubiquitous enzymes which catalyze the breakdown of H_2O_2 in cells and thus control the level of cellular ROS. In some species, the presence of specific catalase enzymes is associated with specific developmental stages or various forms of stress. Their importance in cell function is underscored by recent studies linking the levels of antioxidant enzymes to mutation rate, carcinogenesis and aging [61–64].

The unusual resistance of *Dictyostelium* to UV-light, γ-radiation and chemicals is of considerable interest with respect to DNA repair and cellular response to DNA-damaging agents. Within this context, we have been studying the regulation of genes encoding enzymes required for nucleotide excision repair, one of the main pathways of DNA repair. We demonstrated that these genes are regulated as part of the developmental program, as well as in response to DNA damage by UV-light and the DNA reactive chemotherapeutic agent cisplatin [23,25,26].

The rapid upregulation of NER genes in response to DNA damage suggested that it might be at least partially responsible for the natural resistance of *Dictyostelium* cells to UV and DNA-damaging chemicals. Thus we wished to determine whether the increased resistance of *Dictyostelium* to oxidative agents could also result from the ability of the cells to readily upregulate the antioxidant enzymes, the BER system or the NER system in a manner similar to the response seen with NER genes after exposure to UV and chemicals. To this end we have identified and characterized *Dictyostelium* catalase and superoxide dismutase genes and studied their response to oxidative stress.

We have shown that *Dictyostelium* has two catalases, which is the case in most organisms, except in animals as well as some parasitic bacteria, where only one catalase has been reported [65]. Both *Dictyostelium* catalase genes are highly related to each other, as well as to catalases from other organisms. This is particularly true in the highly conserved 360 amino acid core region [65]. *cat*A encodes a 497 aa polypeptide which is most similar to animal catalases. *cat*B (encoding a 693 aa long polypeptide) is most similar to those bacterial and fungal catalases which are induced by development (sporulation) or stress-related conditions, including stationary phase. These large catalases have a conserved flavodoxin-like C-terminal extension, which has a stabilizing effect on the entire protein, enabling the protein to withstand temperatures of up to 70°C, as well as a variety of denaturing agents [65,66].

Our study revealed unique patterns of expression of the two *Dictyostelium* catalase genes. *cat*A is expressed throughout growth and development. In contrast, *cat*B expression is limited to the terminal stages of morphogenesis. Analysis of separated cell types showed that CatB enzyme activity is exclusively found in prespore cells, while CatA enzyme activity is found only in prestalk cells. Moreover, the enzymes were found to reside in different cellular compartments within their respective cell types. Differential regulation of catalase expression during development has been observed in other eukaryotic organisms, including the conidia specific catalases of *E. nidulans* and *N. crassa* [48,67–69]. However, to our knowledge, this level of temporal and spatial complexity of the *Dictyostelium* catalase enzymes is unprecedented. We suggest that the accumulation of the CatB enzyme in prespore cells ultimately affords the spores protection from oxidation during prolonged dormancy. In addition, our observation that disruption of the *cat*B gene can suppress the aggregationless phenotype of the *car*1/*car*3 double mutant, suggests that ROS play an important role in morphogenesis (unpublished).

The unexpected finding that CatA is localized to the prestalk cells raises questions of whether all, or only a fraction of the cells at the onset of development express *cat*A. If all the cells are expressing the gene, then expression would have to become limited to the prestalk cells as development proceeds. Alternatively, the expression of catalase may be linked to the stage of the cell cycle of individual cells at the onset of development, which is a factor in the determination of cell fate [70,71]. This interesting question awaits further study.

To test whether antioxidant genes and/or enzyme levels are rapidly upregulated in *Dictyostelium* in response to oxidative stress, we treated cells with H_2O_2. We show here that the *Dictyostelium* antioxidant genes *cat*A, *cat*B and *sod*A (the Cu/Zn superoxide dismutase) are not induced by H_2O_2 in growing cells. Induction of antioxidant enzymes to varying degrees by H_2O_2 has been reported in other systems, including *Candida albicans* [72], rat lens [73], primary rat hepatocytes [74] or the developmentally regulated catalase of *E. nidulans* [68], so it was surprising that in *Dictyostelium* neither *cat*A nor *cat*B were induced by H_2O_2. Perhaps this is due to the fact that there is a high level of CatA enzyme activity in growing cells of *Dictyostelium*. In addition, it is possible that transcription factors expressed only in development are required for H_2O_2 induction of *cat*B.

We also examined the response of the oxidative damage repair gene (*ape*A) to a sudden burst of H_2O_2. This gene encodes the BER apurinic/apyrimidinic (AP) endonuclease, which cleaves the abasic sites generated after removal of oxidized bases by specific DNA glycosylases/AP lyases. In humans [75,76] and bacteria [77] AP enzymes have been observed to be induced by sublethal levels of ROS. However, the *Dictyostelium ape*A was not induced by H_2O_2. The above experiments suggest that the increased resistance of *Dictyostelium* to oxidative agents is not due to a rapid mobilization of antioxidants or BER enzymes.

There is accumulating evidence for the involvement of ROS in UV-induced cellular and DNA damage. It has been demonstrated that each region in the UV spectrum induces the formation of the oxidatively modified base, 8-oxo-7,8-dihydro-2′-deoxyguanosine via the formation of different ROS [78]. Moreover, endogenous and exogenous antioxidant enzymes and molecules have been shown to play protective roles in cells exposed to UV [79–83]. The availability of the *cat*A mutant allowed us to check how *Dictyostelium* cells respond to UV in the presence or absence of a mechanism to control bursts of H_2O_2. We also examined the role of antioxidant genes in the *Dictyostelium* response to UV, by analyzing their expression after UV treatment. We found that both catalases and the Cu/Zn SOD genes are not induced by UV. Wild type and *cat*A mutant cells showed similar sensitivity to UV, although sensitivity to H_2O_2 was increased 160-fold in the mutant. Thus, sensitivity to oxidative stress did not result in a parallel sensitivity to UV-light, indicating that the response to UV-light is independent from the response to ROS.

In contrast, the BER enzyme AP endonuclease was rapidly and robustly induced by UV (our results and [28]). The two main repair pathways to reverse DNA damage in eukaryotes are BER and NER. BER functions continuously to rectify DNA damage caused by hydrolysis, ROS and simple alkylating agents. Depending upon the type of base modification, damage can be removed via three distinct BER mechanisms: a single nucleotide patch repair pathway initiated by glycosylases lacking AP lyase activity; a single nucleotide patch repair pathway initiated by glycosylases with AP lyase activity; and a long patch repair pathway in which multiple nucleotides are excised [2]. Common to all three variations is the AP endonuclease. NER is predominantly involved in the removal of DNA damage mediated by UV-light and is specifically responsible for the removal of thymine dimers, 6–4 photoproducts and bulky chemical adducts [84]. The polypeptides involved in NER are for the most part different from the ones utilized by BER. However, there is evidence that two enzymes, XPG and PCNA, function in both pathways [2,84,85]. Though AP endonuclease is not a component of NER, it is of considerable interest that in *Dictyostelium, ape*A mRNA is upregulated in response to UV-light. This is in contrast to the human APE-1 which is not responsive to UV-light and alkylating agents [75]. Paradoxically, *ape*A is not upregulated following treatment with H_2O_2 in *Dictyostelium*, whereas the human APE-1 is upregulated by H_2O_2 and other ROS [75]. The increase in AP-endonuclease and presumably activation of BER following UV treatment may be a contributing factor to the relative resistance of *Dictyostelium* to UV-light and may be another

example of cross talk between defense mechanisms in *Dictyostelium*.

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