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Regulation of the ribonucleotide reductase small subunit gene in *Dictyostelium discoideum*

Pascale Gaudet

A Thesis

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ABSTRACT

Regulation of the ribonucleotide reductase small subunit gene in *Dictyostelium discoideum*

Pascale Gaudet, Ph. D.
Concordia University, 2001

Ribonucleotide reductase catalyzes the reduction of ribonucleotides to deoxyribonucleotides, providing precursors for the synthesis of DNA. Expression of ribonucleotide reductase is correlated with DNA synthesis: it is up-regulated during the DNA synthesis phase of the cell cycle and in the course of DNA repair.

We have examined the regulation of expression of the ribonucleotide reductase small subunit gene of *Dictyostelium discoideum*, *rnrB*, during the cell cycle, in response to DNA-damaging agents and during development. Our results suggest that *rnrB* is expressed during two periods of the cell cycle in *Dictyostelium*, with one expression peak in mid-G2 and one in late G2. A *cis*-acting element referred to as box A appears to be able to confer cell-cycle-regulated expression.

We have shown that the level of *rnrB* transcript increases when cells are treated with mutagens and with hydroxyurea, an inhibitor of ribonucleotide reductase. The response is rapid, transient and independent of protein synthesis. A DNA fragment consisting of the 450 bp upstream of the start codon of *rnrB* has been shown to be sufficient to confer DNA-damage inducibility on heterologous genes. We have used deletion analysis to define the *cis*-acting elements of the *rnrB* promoter required for the response to two different DNA-damaging agents, methyl methane sulfonate and 4-nitroquinoline-1-oxide. Our results indicate that box C can confer response to both drugs,

while box A and box D confer response to methyl methane sulfonate and 4-nitroquinoline-1-oxide, respectively. We have studied the phenotype of a mutant in which part of the *rnrB* promoter has been deleted by gene replacement. The mutant strain fails to up-regulate the *rnrB* gene in response to DNA-damaging agents. This mutant displays increased sensitivity to mutagens as well as prolonged cell cycle arrest upon exposure to mutagens.

Our laboratory has shown by histochemical staining that the *rnrB* gene is expressed only in the posterior, prespore zone during development. We have identified by deletion analysis and site-directed mutagenesis *cis*-acting elements responsible for cell-type-specific expression of *rnrB* during development. Preventing the expression of *rnrB* does not appear to cause morphological defects in *Dictyostelium* development. Using electrophoretic mobility shift assays, we have detected cellular factors that may regulate the expression of the *rnrB* gene.

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-Bonfils, C., Gaudet, P. and Tsang, A. (1999) Identification of *cis*-regulating elements and *trans*-acting factors regulating the expression of the gene encoding the small subunit of ribonucleotide reductase in *Dictyostelium discoideum. J. Biol. Chem.*, **274**, 20384-20390.

CONTRIBUTION OF AUTHORS

All the data presented in this thesis is the work of the author, except for the following:

-The *rnrB* promoter was sequenced by Dr. Caroline Grant (Tsang *et al.*, 1996).

-Sequencing of the *rnrB* promoter mutants was done by Nathalie Brodeur at the Centre for Structural and Functional Genomics, Concordia University.

-The RnrB-ubi-S65TGFP and RnrB-ile-αpgal constructs were made and transformed into *Dictyostelium* by Dr. Harry MacWilliams.

-Transformation of Δ-280/A1, Δ-280/A2, Δ-450/A1 and Δ-450/A2 constructs into *Dictyostelium* as well as histochemical stainings (Figure 20) were done by Dr. Harry MacWilliams.

-Cell cycle synchronizations, BrdU assays, β-galactosidase assays and RNA extraction for synchronized cells were done by Dr. Harry MacWilliams.

-β-galactosidase assays (Figure 12) were done by Dr. Harry MacWilliams.

-Deletions of the *rnrB* promoter were made in collaboration with Claire Bonfils, Zeina Saikali and Abraham Shtevi.

-Developmental stainings (Figure 19) done by Claire Bonfils and pictures were taken by Dr. Adrian Tsang.

TABLE OF CONTENTS

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xiv

1. INTRODUCTION 1

1.1. Cell cycle control of gene expression 2

1.2. Cellular responses to DNA damage 3

1.2.1. Cell-cycle arrest 4

1.2.2. Apoptosis 6

1.2.3. Modifications in gene expression during genotoxic stress 7

1.2.4. Mechanisms mediating the transcriptional response to DNA damage 9

1.2.5. Ribonucleotide reductase expression in response to DNA damage 14

1.3. Developmental control of gene expression in *Dictyostelium* 16

1.3.1. Role of ribonucleotide reductase during *Dictyostelium* development 17

1.3.2. Regulation of cell-type specific gene expression in *Dictyostelium* 17

2. MATERIALS AND METHODS 20

2.1. Growth, development and transformation of *Dictyostelium* cells 20

2.2. Generation of deletions of the *rnrB* promoter 20

2.3. Site-directed mutagenesis 23

2.4. Disruption of the *rnrB* promoter by homologous recombination 26

2.5. Treatment of *Dictyostelium* cells with drugs and cell survival assays 26

2.6. RNA analyses 28

2.7. Assay for β-galactosidase 31

2.8. Histological stainings 31

2.9. Electrophoretic mobility shift assays (EMSAs) 32

TABLE 1. Sequence of the synthetic oligonucleotides used for EMSAs 34

3. RESULTS 35

3.1. Defining the *rnrB* promoter 35

3.2. Expression of the *rnrB* gene is cell cycle-regulated 35

3.2.1. Role of box A in regulating the cell-cycle expression of *rnrB* 38

3.3. Response of the *rnrB* gene to DNA-damaging agents 42

3.3.1. DNA-damaging agents stimulate the accumulation of *rnrB* transcript 43

3.3.2. Hydroxyurea causes up-regulation of *rnrB* expression in vegetative cells 45

3.3.3. Effect of DNA-damaging agents on *rnrB* expression is independent of developmental stage 45

3.3.4. Up-regulation of *rnrB* is independent of protein synthesis 48

3.3.5. Conferring DNA-damage inducibility on heterologous genes 50

3.3.6. Time course of the DNA-damage response 52

3.3.7. *rnrB*-driven gene induction results in up-regulation at the protein level 52

3.3.8. Identification of *cis*-acting elements controlling the DNA damage response 55

3.3.9. Construction of a mutant defective in the *rnrB* response to mutagens 61

3.3.10. Physiological effects of a genomic deletion of the DNA-damage response elements 62

3.4. Developmentally-regulated expression of *rnrB* 71

3.4.1. Identification of *cis*-acting elements controlling the developmental expression of *rnrB* 71

3.4.2. Mutational analysis of the prespore-specific element box A 74

3.4.3. Effect of a genomic deletion of the cell-type-specific response elements 74

3.5. Factors regulating the expression of *rnrB* 78

3.5.1. GBF, a known transcription factor, does not bind the *rnrB* promoter 78

3.5.2. Cellular factors binding to box A 80

3.5.3. Cellular factors binding to box B 83

4. DISCUSSION 88

4.1. Cell cycle regulation of *rnrB* 88

4.2. Response of *rnrB* to DNA-damaging agents 89

4.2.1. Change in *rnrB* transcript level in response to DNA-damaging agents 89

4.2.2. Response of *rnrB* to the inhibitor hydroxyurea 90

4.2.3. Regulation of the DNA-damage response 91

4.2.4. The *rnrB* promoter as an inducible expression system for *Dictyostelium* 92

4.2.5. Promoter elements driving the DNA damage response in *rnrB* 94

4.2.6. Physiological role of the DNA damage induction 95

4.3. Developmental regulation of *rnrB* 97

4.3.1. Elements conferring cell-type-specific expression 97

4.3.2. Trans-acting factors controlling developmental expression 99

4.4. Box A may be a general repressor 100

5. CONCLUSIONS 102

6. REFERENCES 103

LIST OF FIGURES

FIGURE 1. Strategy for site-directed mutagenesis of box A of the *rnrB* promoter. 25

FIGURE 2. Strategy for replacement of the *rnrB* promoter by the blasticidin-resistance gene. 27

FIGURE 3. 5' upstream region of *rnrB*. 36

FIGURE 4. Cell cycle regulation of *rnrB* after synchronization. 39

FIGURE 5. Cell-cycle regulated expression directed from wild-type and mutated versions of box A 41

FIGURE 6. Effect of DNA-damaging agents on the accumulation of the *rnrB* transcript.. 44

FIGURE 7. Effect of hydroxyurea on the accumulation of the *rnrB* transcript 46

FIGURE 8. Regulation of *rnrB* by DNA-damaging agents during growth and development. 47

FIGURE 9. Effect of 4NQO and cycloheximide on the accumulation of the *rnrB* transcript. 49

FIGURE 10. Effect of DNA-damaging agents on the accumulation of the *rnrB* and GFP transcripts during growth and development. 51

FIGURE 11. Time course of DNA-damage induction of the *rnrB* and GFP genes. 53

FIGURE 12. ß-galactosidase activity of AX2 cells transformed with a RnrB-ile-αpgal fusion construct upon treatment with DNA-damaging agents. 56

FIGURE 13. Transcriptional response directed by the deletion constructs in the presence of DNA-damaging agents. 59

FIGURE 14. Southern blot showing that the *rnrB* promoter locus has been replaced by the BSR marker. 63

FIGURE 15. Effect of DNA-damaging agents on the accumulation of the *rnrB* transcript in RnrB-P-KO mutants. 64

FIGURE 16. Survival of the RnrB-P-KO strain to DNA-damaging agents. 65

FIGURE 17. Growth rates of the RnrB-P-KO mutant strain and the control strain following treatment with MMS. 67

FIGURE 18. Number of viable cells in RnrB-P-KO and control cultures treated with MMS. 69

FIGURE 19. Histochemical staining of β-galactosidase activity of *Dictyostelium* cells transformed with various RnrB-lacZ constructs 72

FIGURE 20. Histochemical staining of β-galactosidase activity of cells transformed with constructs bearing mutations in the box A element. 75

FIGURE 21. Expression of *rnrB* in AX2, RnrB-P-KO and a control strain. 77

FIGURE 22. Electrophoretic mobility shift assay testing the ability of the *rnrB* promoter elements to bind GBF. 79

FIGURE 23. Electrophoretic mobility shift assay with box A in the presence of unlabelled competitors 81

FIGURE 24. Electrophoretic mobility shift assay showing the developmental regulation of the box A binding factor. 82

FIGURE 25. Electrophoretic mobility shift assays with box A in the presence of unlabelled A1 and A2. 84

FIGURE 26. Electrophoretic mobility shift assay with box B in the presence of unlabelled competitors..... 85

FIGURE 27. Electrophoretic mobility shift assay showing the activity of the box B binding factor during the *Dictyostelium* life cycle. 86

FIGURE 28. Electrophoretic mobility shift assay showing the developmental regulation of the box B binding factor in nuclear extracts. 87

LIST OF ABBREVIATIONS

A: Adenine

AT: Ataxia telengiectasia

ATM: Ataxia telengiectasia mutated

AX: axenic

β-gal: β-galactosidase

Bp: Base pairs

BrdU: Bromodeoxyuridine

BRCA: Breast cancer

BSR: Blasticidin S resistance

BSA: Bovine serum albumin

C: Cytosine

CAE: C/A-rich element

CHX: Cycloheximide

DAPI: 4, 6-Diamidino-2-phenylindole

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol-bis(β-aminoethyl ether)

EMSA: Electrophoretic mobility shift assay

G: Guanine

GBF: G-box binding factor

GFP: Green fluorescent protein

HU: Hydroxyurea

KO: Knock-out

MMS: Methyl methane sulfonate

4NQO: 4-Nitroquinoline 1-oxide

PCR: Polymerase chain reaction

PK: Protein kinase

RNA: Ribonucleic acid

RFC: Replication factor C

RLU: Relative light units

RNR: Ribonucleotide reductase

RPA: Replication protein A

SDM: Standard deviation of the mean

SDS: Sodium dedocyl sulfate

STAT: Signal transducer and activator of transcription

T: Thymidine

TCA: Trichloroacetic acid

UV: Ultraviolet

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. INTRODUCTION

All living organisms possess the information necessary for survival and reproduction in their genome. The genome must be replicated for cells to divide; it must also be protected from agents that damage DNA such as radiation, chemicals present in the environment, or cellular metabolites. Synthesis of DNA requires about 10 different proteins, including DNA helicase, primase, DNA polymerase and DNA ligase (reviewed by Stillman in 1994). The enzyme ribonucleotide reductase catalyzes the reduction of ribonucleotides to deoxyribonucleotides, providing precursors for synthesis of DNA. A balanced pool of all four dNTPs is necessary for faithful replication of DNA and for this reason the expression and activity of ribonucleotide reductase are highly regulated (reviewed in Reichard, 1988). Expression of ribonucleotide reductase is correlated with DNA synthesis. Ribonucleotide reductase is only expressed in actively growing cells and is not expressed in differentiated cells (Engström *et al.*, 1984). Expression of ribonucleotide reductase, as well as several other genes involved in DNA synthesis, peaks in the DNA synthesis (S) phase of the cell cycle. Finally, ribonucleotide reductase expression is increased in response to DNA damage.

The gene that encodes the small subunit of ribonucleotide reductase in *Dictyostelium discoideum*, *rnrB*, has been isolated in our laboratory (Tsang *et al.*, 1996). The work presented in this thesis examines the regulation and the role of ribonucleotide reductase expression during the cell cycle, in response to DNA-damaging agents and during multicellular development in *Dictyostelium*.

1.1. Cell cycle control of gene expression

The cell cycle is divided into four phases: M phase, during which mitosis and cytokinesis take place; G1 (first gap), S phase, characterized by replication of the genome, and G2 (second gap). Cell cycle progression is mediated by cyclins and cyclin-dependent kinases (CDKs). The activity and substrate-specificity of the CDKs are regulated by cyclins. The levels of the different cyclins vary during the cell cycle and are regulated transcriptionally and post-translationally by protein turnover. In mammalian cells, cyclins A and B are involved in regulation of G2 events (B-type cyclins in yeast), while cyclins C, D and E are responsible for progression through G1 (cyclins 1, 2 and 3 in yeast). Degradation of the cyclins is mediated by the ubiquitin pathway (Lodish *et al.*, 1999).

In *S. cerevisiae*, a complex composed of the Cdc28p kinase and Cln3p activates two replication factors in late G1, SBF and MBF (composed of Swi4p/Swi6p and Mbp1p/Swi6p, respectively), that regulate transcription of the *CLN1* and *CLN2* genes as well as other genes required for DNA replication, including DNA polymerase and DNA ligase (Lodish *et al.*, 1999). During S-phase, interaction of Cdc28p with B-type cyclins stimulates the initiation of DNA replication. B-type cyclins Clb1p and Clb2p are complexed with Cdc28p during the G2 phase of the cell cycle and promote entry into mitosis. Similar events take place in other eukaryotes.

In mammalian cells, expression of S-phase genes is controlled by the transcription factors E2F, which are negatively regulated by the retinoblastoma (Rb) gene product. In late G1, Rb is phosphorylated by cdk4-cyclin D and cdk2-cyclin E (G1

cyclins), thereby releasing E2F factors and allowing transcription to take place (reviewed by Kohn in 1999).

The *Dictyostelium* cell cycle is strikingly different from that of other well-characterized eukaryotes in that no G1 phase is detectable. DNA synthesis (S phase) takes place immediately after cell division (M phase), so that most of the cell cycle is G2 (Weijer *et al.*, 1984). How *Dictyostelium* cells monitor cell cycle progression remains to be elucidated.

The absence of a G1 phase brings forward the question of how expression of the "G1 genes" in *Dictyostelium* is regulated. The study of ribonucleotide reductase expression is particularly interesting, because this gene is expressed in G1/S in other species. One possibility is that the *Dictyostelium* G2 phase is divided into subphases that are analogous to G1 and G2 in mammalian cells and yeast. Alternatively, the genes expressed in G1 could be expressed during the M phase, which in *Dictyostelium* precedes the S phase.

1.2. Cellular responses to DNA damage

The preservation of genome integrity is of crucial importance for the survival of any living cell. The presence of an intact genetic code ensures that the cell encodes functional proteins, and that it transmits the correct genetic information to its progeny. For these reasons, a large number of cellular pathways are aimed at responding to chemical and physical modifications to the genome. In eukaryotes, DNA damage can cause cell-cycle arrest, changes in gene expression, as well as apoptosis.

1.2.1. Cell-cycle arrest

In the presence of DNA damage, cells stop dividing for a certain period of time. Cell-cycle arrest is believed to be required to allow DNA repair to occur before chromosome replication or segregation takes place, thereby reducing the likelihood of transmitting erroneous information to the progeny of the damaged cell. In *S. cerevisiae*, several factors involved in recognition and transduction of the DNA damage signal have been identified. Recognition of DNA damage leading to cell-cycle arrest appears to be performed by DNA-binding proteins. Examples of these factors include DNA polymerase ε (Navas *et al.*, 1996), the product of the *RAD17* gene, that bears similarity to a 3' to 5' DNA exonuclease (Lydall and Weinert, 1995, 1997), as well as Rfc5p, a component of the replication factor C (Sugimoto *et al.*, 1997).

Using *S. cerevisiae*, screens have been performed to identify mutants that do not undergo cell-cycle arrest in the presence of DNA damage or blocks in DNA replication. The mutants identified in these screens continue to divide in the presence of damaged DNA and die after a few doublings. Genes identified in these screens include *rad9*, *mec1*, and *rad53* (Paulovich and Hartwell, 1995; Weinert *et al.*, 1994; Weinert and Hartwell, 1988, 1990). The products of the *MEC1* and *RAD53* genes are believed to be involved in the transduction of the DNA damage signal because they contain kinase domains (Lydall and Weinert, 1997; Sidorova and Breeden, 1997; Sanchez *et al.*, 1996; Paulovich and Hartwell, 1995). The product of the *RAD9* gene is also thought to be a transducer of the DNA damage signal and has been proposed to act on cell-cycle progression proteins such as Cdc28p (Siede *et al.*, 1993). Rad9p becomes phosphorylated upon DNA damage, and

phosphorylated Rad9p can interact with Rad53p to mediate cell-cycle arrest (Emili, 1998; Sun *et al.*, 1998).

Thus far, few effectors of cell-cycle arrest have been identified in *S. cerevisiae.* One of them is the anaphase inhibitor Pds1p. Progression into anaphase requires the degradation of Pds1p (Cohen-Fix *et al.*, 1996). Cells harbouring mutations in *PDS1* undergo mitosis abnormally following γ-irradiation (Yamamoto *et al.*, 1996). Interestingly, Pds1p is phosphorylated by the Chk1p kinase upon DNA damage in a Mec1p- and Rad9p-dependent manner (Sanchez *et al.*, 1999). Pds1p phosphorylation upon DNA damage renders it more resistant to proteolysis, therefore mediating M phase arrest (Tinker-Kulberg and Morgan, 1999).

In mammals and in *Schizosaccharomyces pombe*, at least one of the DNA-damage checkpoints appears to be mediated by the Chk1 kinase. Chk1 is phosphorylated upon DNA damage, and in turn phosphorylates Cdc25. Phosphorylated Cdc25 is believed to be unable to activate the Cdc2 kinase, preventing entry into mitosis (Liu *et al.*, 2000; Sanchez *et al.*, 1997). This thus connects the DNA-damage checkpoint with the normal cell-cycle progression machinery, as Cdc2 is responsible for G1 and G2 progression.

In mammalian cells, the best characterized cell-cycle control protein is the tumour suppressor protein p53. In the presence of DNA damage, the stability of p53 increases and its activity is enhanced by covalent modification. Activated p53 results in increased expression of the p21/waf1/cip1 gene, which binds to and inhibits cyclin-dependent protein kinases, resulting in G1 arrest (Wang, 1998; Kubbutat and Vousden, 1998; Ko and Prives, 1996; Meyn, 1995).

Another important regulator of cell cycle progression in response to DNA-damaging agents in mammalian cells is the ATM gene product. Dysfunctional ATM protein results in a disease called ataxia telengiectasia (AT), which is characterized by predisposition to cancer and sensitivity to ionizing radiation (Savitsky *et al.*, 1995). ATM has homology to the budding yeast Mec1p, Tel1p and Rad53p proteins, which are involved in the DNA-damage checkpoints (see above). ATM is activated in response to DNA damage and is responsible for activation of p53 in response to certain types of damage.

1.2.2. Apoptosis

In cases of extensive DNA damage, eukaryotic cells undergo apoptosis. This highly coordinated process of cell elimination can mediate the specific removal of damaged cells. The most characteristic events that take place during programmed cell death are chromatin condensation and degradation, as well as condensation of the cytoplasmic contents (Staunton and Gaffney, 1998; Schwartzman and Cidlowski, 1993). Apoptosis requires the expression of specific factors. In mammalian cells, the tumour-suppressor gene p53 appears to play a central role in programmed cell death in response to genotoxic stress. This is supported by the fact that certain cell lines deficient in p53 are more resistant to mutagens (Levine, 1997). In addition, cells deficient in p53 tolerate genetic abnormalities more than cells that possess wild-type p53 (Ko and Prives, 1996; Levine, 1997; El-Deiry, 1998). The p53 gene is mutated in more than half of human tumours (Levine, 1997). Furthermore, mice lacking p53 are more prone to tumors (Donehower *et al.*, 1992). The general consensus is that these phenomena are the consequence of the failure of dysfunctional p53 to induce apoptosis.

The mechanism by which p53 triggers programmed cell death is not known. It has been proposed that the relative amounts of two gene products, bcl-2 and bax, are important for determining the propensity of a cell to undergo apoptosis. Although blc-2 and bax are homologs, they have opposite activities: bcl-2 is an anti-cell death protein, whereas bax promotes apoptosis. High amounts of bax result in a low threshold for the induction of programmed cell death, and vice versa (Sato *et al.*, 1994; Oltvai *et al.*, 1993). The promoter of the bax gene contains p53-binding sites (Miyashita and Reed, 1995). In addition, p53 has negative effects on the expression of the anti-death gene bcl-2 (reviewed by Basu and Haldar, 1998). Therefore, it is plausible that p53 triggers apoptosis by activating the expression of *bax* while lowering that of *bcl-2*.

The ultimate step of programmed cell death is the activation of proteases that mediate the degradation of the cellular contents (reviewed in Favrot *et al.*, 1998; Thornberry and Lazebnik, 1998).

1.2.3. Modifications in gene expression during genotoxic stress

A change in the expression of a number of genes is another primary response of cells to damaged DNA. In bacteria this response is referred to as the SOS response and involves the recA and lexA proteins. The products of the target genes for the recA system are involved in DNA repair, DNA synthesis and inhibition of cell division (Friedberg *et al.*, 1995).

In eukaryotes, many genes whose expression levels are modified by the presence of DNA-damaging agents have been identified. In yeast the genes activated by DNA-damaging agents include those involved in nucleotide excision repair, post-replication

repair, and double-strand break repair. Also induced by DNA-damaging agents are some of the genes thought to play a dual role in nucleic acid metabolism and DNA repair; for example, the genes encoding DNA ligase I, DNA polymerase I, and ribonucleotide reductase (Friedberg *et al.*, 1995).

The development of the microarray technology, which allows the simultaneous monitoring of the expression of thousands of genes, suggests that the transcriptional response to DNA-damaging agents in *S. cerevisiae* may be more complex than previously thought. Samson and colleagues (1999, 2000) have monitored the expression of about 6,200 genes after treatment of *S. cerevisiae* with MMS. They have found that the level of about 5% of the transcripts increase by more than 4-fold (Jelinsky and Samson, 1999) and 10% were induced by 3-fold or more (Jelinsky *et al.*, 2000). In addition to genes involved in DNA repair and DNA synthesis, many other genes were up-regulated, including genes involved in stress response and detoxification, cell cycle control, carbohydrate metabolism, signalling, cell wall biogenesis, and protein degradation. This may be explained by the fact that MMS causes significant damage to proteins, in addition to damaging nucleic acids. The up-regulation of a number of these groups of genes may be caused by "protein-damage response" rather than by "DNA-damage response". These include genes involved in protein degradation and amino acid metabolism, detoxification, and cell wall biogenesis.

Another study that examined the response to several DNA-damaging agents supports this interpretation. *S. cerevisiae* cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), *tert*-butyl hydroperoxide (t-BuOOH), 4NQO or γ irradiation, and the global gene expression profile

was monitored using microarrays. Overall, about one third of all the genes in *S. cerevisiae* were found to respond to one or more DNA-damaging agents. However only 21 genes were regulated by all the agents tested: 12 were consistently up-regulated, and 9 were down-regulated. Genes up-regulated include those encoding the DNA-damage inducible large subunit of ribonucleotide reductase and glutathione transferase. Genes down-regulated include genes coding for histone H2B and RNA helicase (Jelinsky *et al.*, 2000).

The response to DNA-damaging agents in mammalian cells is complex and involves many genes and proteins. These genes and proteins are associated with diverse cellular functions including not only those implicated in DNA repair and its related processes, but also transcription factors, growth factors, growth factor receptors, tumor suppressor proteins, protein kinases, G-protein, responses to tissue injury, inflammation and protective responses, and differentiation-specific proteins (reviewed by Bender *et al.*, 1997). The products of some of these genes are thought to be needed to fuel DNA synthesis during repair. The expression of other factors presumably reflects the requirement for coordination of regulated responses between cells in multicellular organisms.

1.2.4. Mechanisms mediating the transcriptional response to DNA damage

A. Activation of gene expression in the presence of DNA damage

In bacteria, the transcriptional response to DNA-damaging agents is mediated by the lexA and the recA proteins. Under normal conditions, the recA protein is maintained at low levels and lexA represses the expression of the target genes of the SOS response.

In the presence of DNA damage or blocks in DNA replication, the protease function of recA is activated. LexA is cleaved by recA, thus removing it from the promoter of its target genes. Transcription of these genes can then take place (Friedberg *et al.*, 1995).

Eukaryotes appear to lack a generalized response system for DNA damage-induced transcription such as the recA system found in prokaryotes. The promoters of several DNA-damage responsive genes in *S. cerevisiae* have been analyzed. A consensus DNA-damage response element (DRE) has been identified in several of these genes (Liu *et al.*, 1997; Wolter *et al.*, 1996; Singh and Samson, 1995; Sancar *et al.* 1995; Xiao *et al.* 1993; Siede and Friedberg, 1992; Sebastian *et al.*, 1990). In the case of the *RAD2* gene, deletion of DRE1, the *cis*-acting element involved in the DNA damage response, has been shown to have a deleterious effect on survival following treatment with mutagens (Siede and Friedberg, 1992). However, this element is not present in all DNA damage-responsive promoters, and is present in a number of non-inducible genes. A possible explanation for these observations is that the response to DNA-damaging agents has different requirements depending on the promoter.

Several studies have made attempts to identify the transcription factors that are involved in the DNA damage response in yeast. In one of these studies mutants were isolated that constitutively expressed *RNR3*, the gene encoding the DNA-damage inducible large subunit of ribonucleotide reductase (Zhou and Elledge, 1992). This has lead to the identification of *CRT1* (constitutive *RNR3* transcription 1) which encodes a DNA-binding protein. Upon DNA damage Crt1p becomes phosphorylated, which reduces its affinity for its target site and allows increased transcription of its target genes, including *RNR2*, *RNR3* and *RNR4* (Huang *et al.*, 1998). Other *crt* mutants include *TUP1*

and *SSN6*, two other repressors of transcription (Zhou and Elledge, 1992). Crt1p appears to recruit Ssn6p and Tup1p to the promoters of the *RNR2* and *RNR3* genes (Huang *et al.*, 1998).

Other transcription factors that have been associated with the transcriptional response to DNA damage include RPA, or replication protein A, Swi4p and Swi6p, as well as Ume6p. RPA is a multifunctional protein, which has been implicated in many varied processes such as DNA replication, nucleotide excision repair and homologous recombination (Brill and Stillman, 1991; Coverley *et al.*, 1991; Burns *et al.*, 1996; Alani *et al.*, 1992). RPA is phosphorylated in response to DNA damage in a Mec1p-dependent fashion (Brush *et al.*, 1996). RPA has been shown to bind a DNA fragment comprising the consensus DRE1 element described above from the promoters of several DNA repair genes, including *MAG, MGT1* and *PHR1*. Also, RPA was found to bind to the DRE element of several *RAD* genes: *RAD1, RAD2, RAD4, RAD10, RAD16* and *RAD51*. Elements in the promoters of other DNA damage-responsive genes also bind RPA, including *RNR2* and *RNR3* (Singh and Samson, 1995). Not all these genes, however, respond to DNA damage. Another transcriptional factor that has been implicated in the transcriptional response to DNA damage is the Ume6p repressor. Ume6p-binding sites have been found in the promoters of several DNA damage-responsive genes, including *PHR1, RAD2, RAD7*, and *RAD53*. Deletion of the *UME6* gene has been shown to increase sensitivity to UV irradiation (Sweet *et al.*, 1997). The Swi4p and Swi6p transcriptional activators have also been associated with the transcriptional response in the presence of DNA-damaging agents. Cells that have mutations in the *SWI4* or *SWI6* genes have reduced ability to induce *RNR2* and *RNR3* in the presence of DNA-damaging

agents (Ho *et al.*, 1997). Furthermore, *swi6* mutants have higher sensitivity to DNA-damaging agents than wild-type cells (Johnston and Johnson, 1995).

Recently, global changes in gene expression following treatment with several DNA-damaging agents have been monitored using DNA microarrays (Jelinsky *et al.*, 2000). The large amount of data generated by this method allowed grouping of genes that are similarly regulated. The promoters of the members of these groups have been analyzed for the presence of similar *cis*-acing elements. One such element is found in a group comprising the *MAG1* gene, encoding a methyl-DNA-glycosylase known to repair lesions caused by MMS. This element is the target site for the transcription factor Rpn4p. Interestingly, deletion of the *RPN4* gene rendered many of the genes of that group unresponsive to MMS, without having any effect on genes of other clusters. Rpn4p is known to regulate expression of genes encoding proteins involved in protein degradation, suggesting that it is involved in the protein damage response pathway rather than in the DNA-damage response pathway. Other known binding motifs that have been recognized include those of the Rap1p and the Ste12p proteins, regulating the expression of genes encoding ribosomal proteins and proteins required for mating, respectively. These factors are unlikely to be responsible for the DNA-damage response. Binding sites for the DNA-damage-specific factor Crt1p have not yet been identified using this method.

B. Signal transduction cascade leading to the DNA-damage-induced transcriptional response

The signal transduction pathway leading to gene activation in response to DNA-damaging agents has also been analyzed in yeast. Cells carrying mutations in the *POL2*

gene, encoding DNA polymerase ε, as well as in the *MEC1* and *RAD53* genes, encoding kinases, are defective in the induction of *RNR3*, one of the genes that encode the large subunit of ribonucleotide reductase in yeast (Navas *et al.*, 1995, 1996). *rad9* mutants are also defective in *RNR3* induction, as well as for induction of several other DNA damage-responsive genes, including *RNR1*, *RNR2*, *CDC9*, *DUN1*, *RAD51* and *RAD54* (Aboussekhra *et al.*, 1996). Pol2p, Mec1p, Rad53p and Rad9p also appear to be involved in cell-cycle arrest, indicating that they could have a central role in signal transduction during the DNA damage response that leads to cell-cycle arrest as well as changes in gene expression. A protein kinase required for high level of induction of *RNR2* and *RNR3* by DNA damage in yeast, Dun1p, has been identified. Dun1p, however, is not required for induction of *UBI4* and *DDR48*, two other DNA damage-responsive genes, suggesting that more than one pathway is responsible for DNA damage induction in yeast (Zhou and Elledge, 1993).

The pathway mediating the DNA damage-induced transcriptional response in mammalian cells appears to be very complex. It is believed to comprise general signal transducers such as JNK, EKR, p38 MAP, MEK kinases, ras, src (reviewed by Bender *et al.*, 1997; Firtz and Kaina, 1997) as well as transcription factors such as c-Jun and CREB (Bender *et al.*, 1997). However, it is not known whether any of these factors is directly involved in the response. Transcription factors ultimately recruited by this response are thought to include p53 and the breast cancer susceptibility gene BRCA1.

One of the main transcriptional activators known to cause increased expression of DNA damage-responsive genes is p53 (reviewed by Lohrer, 1996). The signal transduction pathway leading to p53 activation is very complex. Normally, p53 has a

relatively short half-life, and is targeted for ubiquitin-mediated degradation by the MDM2 protein, which has a ubiquitin-ligase activity. In the presence of DNA-damage, p53 is phosphorylated by several kinases, including ATM (a homolog of the *S. cerevisiae TEL1* gene), ATR, DNA-PK, JNK and the Chk2 kinase (a homolog of the *S. cerevisiae RAD53* gene) (Lohrum and Vousden 1999; Giaccia and Kastan, 1998; Hirao *et al.*, 2000). These modifications reduce the affinity of MDM2 for p53. As a result, p53 becomes more stable and increases in concentration. MDM2 can also be phosphorylated by DNA-PK, and this also causes a reduction in its binding affinity for p53.

The histone acetyl transferases p300 and PCAF can acetylate p53 in the presence of DNA damaging agents, which activates the transcriptional function of p53 (Liu *et al.*, 1999; Sakaguchi *et al.*, 1998). This acetylation stabilizes p53 in a MDM-2 independent manner (Yuan *et al.*, 1999).

BRCA1 is phosphorylated upon DNA damage (Scully *et al.*, 1997) and has been shown to physically interact with p53 and to increase its transcriptional activity *in vitro* (Zhang *et al.*, 1998).

1.2.5. Ribonucleotide reductase expression in response to DNA damage

The enzyme ribonucleotide reductase catalyzes the first reaction in *de novo* DNA synthesis, the conversion of ribonucleotides to deoxyribonucleotides (Reichard, 1988). Because of its essential role in DNA synthesis, this enzyme plays an important part in the repair of damaged DNA. The expression of the genes encoding both subunits of ribonucleotide reductase is increased in cells treated with DNA-damaging agents in *E. coli*, *S. cerevisiae*, mammalian cells (reviewed by Elledge *et al.*, 1993) as well as in

Dictyostelium discoideum (Gaudet and Tsang, 1999; our laboratory, unpublished observations). That induction of ribonucleotide reductase by DNA-damaging agents is observed in all species studied so far underscores the importance of this response. Another indication of the importance of overexpression of ribonucleotide reductase in the DNA damage response is the observation that it is one of only 12 genes out of 6,200 transcripts studied in *S. cerevisiae* found to be up-regulated in the presence of 6 different DNA-damaging agents (Jelinsky *et al.*, 2000).

A number of studies directly suggest that increased ribonucleotide reductase expression is advantageous to cells with damaged DNA. Preventing the up-regulation of one of the genes encoding the small subunit of ribonucleotide reductase following DNA damage causes increased cell death and slows down DNA repair in mammalian cells (Tanaka *et al.*, 2000). Also, an increase in the number of chromosome aberrations was observed when irradiated human lymphoblastoid cells were incubated with ribonucleotide reductase inhibitors, such as hydroxyurea (Antoccia *et al.*, 1994; Collins and Oates, 1987) or paracetamol (Honglso *et al.*, 1993). This effect can be mimicked by deoxyribonucleoside depletion (Hunting and Dresler, 1985), and reversed if all four dNTPs are provided (Honglso *et al.*, 1993). It has also been shown that a proper balance of dNTPs is important for accurate repair (Holmberg, 1989).

Inappropriate ribonucleotide reductase expression has been implicated in carcinogenesis. Transformed cells express high levels of ribonucleotide reductase (Elford *et al.*, 1970). The expression of this enzyme can be altered by tumour promoters as well as transforming growth factor β_1 (Hurta and Wright, 1992; Hurta *et al.*, 1991). In the presence of activated oncogenes, overexpression of the small subunit of ribonucleotide

reductase has been shown to affect the rates of tumour formation and metastasis in mice (Fan *et al.*, 1996, 1997). Inhibitors of ribonucleotide reductase have been shown to slow the growth of tumor cells. For these reasons, ribonucleotide reductase is a key target for chemotherapeutic drugs (reviewed in Szekeres *et al.*, 1997). The factors that regulate the expression of ribonucleotide reductase are potential targets for the design of new chemotherapeutic drugs.

The study of the regulation of the ribonucleotide reductase genes has been complicated by the cell-cycle-dependent expression of ribonucleotide reductase. Thus analysis of the effects of DNA-damaging agents in proliferating cells may be complicated by mechanisms that overlap the repair and growth processes. The developmental phase of the *Dictyostelium* life cycle allows the study of DNA-damaging agents on gene expression in the absence of cell growth.

1.3. Developmental control of gene expression in *Dictyostelium*

The asexual life cycle of *Dictyostelium discoideum* consists of two mutually exclusive phases. When nutrients are abundant, *Dictyostelium* grows vegetatively as single-celled amoebae that divide by binary fission. Upon depletion of the food source, the amoebae aggregate to form multicellular structures consisting of approximately 10^5 cells which ultimately form fruiting bodies made up of 20% stalk cells and 80% spore cells. Completion of the developmental program takes approximately 24 hours. During the developmental phase, *Dictyostelium* cells come together to form multicellular aggregates 8 h after the initiation of development. By 16 h the multicellular aggregates called slugs are differentiated along the anterior-posterior axis. Prestalk cells occupy the

anterior one-quarter of the slug and prespore cells are located in the posterior three-quarters. These precursor cells ultimately differentiate into the stalk cells and spores of the mature fruiting body. Prespore and prestalk cells can be classified with respect to the gene markers they express (reviewed by Loomis in 1996).

1.3.1. Role of ribonucleotide reductase during *Dictyostelium* development

At the slug stage of *Dictyostelium* development, cells in the prespore region undergo a wave of DNA synthesis (Zimmerman and Weijer, 1993; Shaulsky and Loomis, 1995; Deering, 1982; Durston and Work, 1978; Zada-Hames and Ashworth, 1978). The role of this developmentally programmed burst of DNA synthesis is unknown. It has been suggested by different investigators to fuel cell division (Zimmerman and Weijer, 1993; Durston and Work, 1978; Zada-Hames and Ashworth, 1978), mitochondrial replication (Shaulsky and Loomis, 1995), or both (Deering, 1982). Temporally and spatially correlated with this wave of DNA synthesis is the elevated expression of the gene encoding the small subunit of ribonucleotide reductase, *rnrB* (Tsang *et al.*, 1996). As in other organisms, it appears that fluctuations in the expression of *rnrB* can be used to predict changes in the rate of DNA synthesis. It is therefore possible that altering the pattern of *rnrB* expression may be used as a tool to change the profile of DNA synthesis in evaluating the role of DNA synthesis in development.

1.3.2. Regulation of cell-type specific gene expression in *Dictyostelium*

An important question in developmental biology is to understand the factors that control cellular differentiation. Cells of different types express different genes, and one

approach to understand how cells differentiate is to study the factors that regulate cell-type-specific gene expression. Manipulating the regulatory regions of promoters provides a convenient way of changing the pattern of gene expression.

The regulatory regions of several genes that are expressed predominantly in prespore cells have been characterized. Most of these promoters contain consensus C/A-rich elements, called CAEs, which have been shown to be important for transcriptional activity (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al.*, 1994; Haberstroh *et al.*, 1991; Haberstroh and Firtel 1990; Fosnaugh and Loomis, 1993). Also required is an A/T-rich element located downstream of the CAEs (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al.*, 1994). When joined with a heterologous basal promoter, neither the CAEs nor the A/T rich element alone is able to drive expression in prespore cells. However, expression in prespore cells can be stimulated when the CAEs and the A/T-rich element are placed together with a heterologous basal promoter (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al.*, 1994). The CAEs exhibit strong affinity for the developmentally regulated transcriptional factor GBF (Schnitzler *et al.*, 1994). Cells carrying a null mutation in the gene encoding GBF are arrested at the loose aggregate stage, *before* cell differentiation has occurred (Schnitzler *et al.*, 1994), implying that besides the interaction between GBF and CAEs, prespore gene expression requires the interaction of other factors and regulatory elements.

A *cis*-acting element that is responsible for prestalk-specific gene expression has been identified in the promoters of the prestalk-specific genes *ecmA* and *ecmB* (Harwood *et al.*, 1993). The factor that binds to this sequence, Dd-STATa, has been isolated and characterized (Kawata *et al.*, 1997). Dd-STATa is a member of the STAT family of

transcriptional regulators, which in mammalian cells are involved in responses to cytokines (reviewed by Darnell in 1997). DdSTAT controls cell-type-specific gene expression through repression of expression in other cell types (Mohanty *et al.*, 1999). The expression of *cudA* in pretalk cells is also regulated by DdSTAT (Fukuzawa and Williams, 2000)

The regulation of *rnrB* appears to be more complex than that of the other known prespore genes. In addition to expression in prespore cells, it is expressed during vegetative growth. A cursory examination of the G/C-rich sequences in the promoter region of *rnrB* shows the absence of known *cis*-acting elements. Only one G/C-rich sequence in the promoter of *rnrB* exhibits similarity to half a C/A-rich element. We have shown by deletion analysis that expression of *rnrB* in vegetative cells does not require any of the G/C-rich sequences found in the promoter. In addition, we have identified two G/C-rich sequences that can direct prespore expression during postaggregative development. Results from electrophoretic mobility shift experiments suggest that these two G/C-rich sequences interact with factors that are distinct from the transcriptional factor GBF (Bonfils *et al.*, 1999). Characterization of the *rnrB* promoter may reveal novel factors involved in prespore-specific gene expression.

2. MATERIALS AND METHODS

2.1. Growth, development and transformation of *Dictyostelium* cells

Cells of the axenic strain AX2 were grown either axenically in HL5 medium (Ashworth and Watts, 1970) or on lawns of *Enterobacter aerogenes* on SM agar (Sussman, 1966). At the logarithmic phase, 2-4 x 10^6 cells/ml in HL5 or when the bacterial lawns began to clear, the cells were harvested and washed in ice-cold KKP buffer (20 mM KH_2PO_4/K_2HPO_4, pH 6.2), and allowed to develop on a solid substratum as described previously (Bonfils *et al.*, 1994).

Plasmid DNAs were introduced into AX2 cells by calcium phosphate coprecipitation as described previously (Early and Williams, 1987) or by electroporation (Tuxworth *et al.*, 1997). Transformants were selected in HL5 containing 20 μg/ml G418 (Life Technologies) or 10 μg/ml blasticidin S (ICN) as appropriate.

2.2. Generation of deletions of the *rnrB* promoter

The *Xba*I/*Bam*HI genomic fragment (Grant *et al.*, 1990) contains two-thirds of the coding region and 450 bp of 5' noncoding region of *rnrB*. This fragment was cloned in-frame to *lacZ* into the *Xba*I/*Bgl*II sites of pDdGal16 (Harwood and Dury, 1990) to generate construct Δ-450. To construct the other 5' deletions, sequences were progressively removed with Bal31 (Sambrook *et al.*, 1989) from the *Xba*I site. The cleaved DNA fragments were excised with *Bam*HI and cloned into the *Bgl*II site and the end-filled *Hin*dIII site of pDdGal16. The end points of the deletions were determined by sequencing using the primer 5'-GAGAATTGGTTCAATGAATG-3', complementary to positions +26 to +45 of the sense strand of *rnrB*. With the exception of Δ-450, all the 5'

deletion constructs retained the *Xba*I-*Kpn*I-*Eco*RI multiple cloning site of pDdGal16. The 5' deletion constructs are designated Δ-y, where y refers to the nucleotide at the 5' deletion end-point. Base +1 is the A residue in the initiation codon ATG.

Internal deletions were constructed using two different PCR products of the *rnrB* promoter. The 5' primer for both products was 5'-TTACTAGTGAAATACCTGCACCTCC-3', where the underlined base corresponds to a mismatch in the primer to its complementary sequence that generates a *Spe*I site to allow cloning in the *Xba*I site of the deletion constructs. This primer is located in the *capA* open-reading frame, from base -1779 to base -1755 with respect to the A of the first ATG of *rnrB*. The sequences of the two 3' primers are as follows: box B primer: 5'-TTGAATTCAAAATACACACACATTCCCG-3', and box C primer: 5'-TTGAATTCATGATGGAATCACCGTTCC-3'. The engineered *Eco*RI sites in these primers, as shown by the underlined bases, were used for cloning in the deletion constructs. Polymerase chain reactions were performed with Expand™ (Boerhinger Mannheim) according to the manufacturer's instructions, using an annealing temperature of 55°C. The internal deletions are designated -XΔ-Y, where X and Y indicate the nucleotides 5' and 3' from the deleted regions, respectively. All internal deletions retain the *Eco*RI site from the polylinker of the vector. For construct -444Δ-212, one of the PCR products was digested with *Spe*I and *Xba*I and inserted into the *Xba*I site of construct Δ-212.

The constructs used for testing the response to DNA-damaging agents were made as follows. Deletions -429Δ-340, -429Δ-280 and -429Δ-212 were constructed using the PCR product generated with the 3' box C primer, digested with *Spe*I and *Kpn*I, and

inserted into the *Xba*I and *Kpn*I sites of the 5' deletion constructs Δ-340, Δ-280 and Δ-212, respectively. Deletions -444Δ-311 and -444Δ-280 were obtained by inserting the *Spe*I/*Xba*I fragment of the above PCR product into the *Xba*I site of Δ-311, Δ-280 and Δ-212, respectively. Deletions -359Δ-280 and -359Δ-212 were produced with the same PCR fragment digested with *Spe*I and *Eco*RI and cloned into the *Xba*I and *Eco*RI sites of constructs Δ-280 and Δ-212, respectively. Finally, deletion -292Δ-212 was constructed with the PCR product obtained with the 3' box B primer, digested with *Spe*I and *Eco*RI, and inserted into the *Xba*I and *Eco*RI sites of the deletion construct Δ-212 (Gaudet and Tsang, 1999).

The constructs used for testing the developmental expression directed by the *rnrB* promoter were constructed in a similar way, except that the PCR products were digested with *Xba*I rather than *Spe*I, which removed all the sequences upstream from the *Xba*I site of the *rnrB* promoter, located 450 bp upstream from the ATG site.

For the constructs containing individual G/C-rich boxes in Δ-212, pairs of oligonucleotides were designed in such a way that, after annealing, there would be on both sides overhanging ends, GATC, that are compatible with a *Bgl*II site. The sequences of the oligonucleotides for reconstituting the boxes are as follows: for box B, 5'-GATCCTTTCGGGAATGTGTGTGTATTA-3' and 5'-GATCTAATACACACACATTCCCGAAAG-3'; for box C, 5'-GATCCATTGGAACGGTGATTCCATCAA-3' and 5'-GATCTTGATGGAATCACCGTTCCAATG-3'; and for box D: 5'-GATCCTCTAGAATCGGAGTGGTACCCAAAA-3' and 5'-GATCTTTTGGGTACCACTCCGATTCTAGAG-3'. The recipient plasmid, Δ-212, was

modified to accommodate the GATC overhang of the annealed oligonucleotides by replacing the *Xba*I-*Kpn*I-*Eco*RI sites with *Spe*I-*Bgl*II-*Eco*RI, obtained from the multiple cloning site of the vector pPC86 (Chévray and Nathans, 1992).

2.3. Site-directed mutagenesis

Box A of the *rnrB* promoter was mutagenized using the strategy shown in Figure 1 (Higuchi, 1990). These constructs were made using a modified β-galactosidase reporter, ile-αpgal, that is more active and more labile than the version present in the pDdGal16 vector. The reporter is a further development of the "N-terminal-rule" reporter "ile-gal" (Detterbeck *et al.*, 1994) in which the originally N-terminally truncated beta-galactosidase (Brake *et al.*, 1978) has been replaced with an enzyme containing a complete alpha peptide; it shows 10- to 100-fold increased activity with an unchanged protein halflife (H. K. MacWilliams, personal communication).

To construct Δ-450/A1-ile-αpgal and Δ-450/A2-ile-αpgal, the template was a genomic clone of *rnrB* in BlueScript (Strategene) (Grant *et al.*, 1990). For Δ-280/A1-ile-αpgal and Δ-280/A2-ile-αpgal, the template was the internal deletion -444Δ-280. The sequences of the four primers used for mutagenesis are (1): reverse primer (in BlueScript) 5-AGCGGATAACAATTTCACACAGG-3' (for the Δ-450 constructs) or 5'-CTTGTCTAACACCAGAGTCTG-3' (which anneals to bases -696 to -676 of the *rnrB* promoter) for the Δ-280-ile-αpgal constructs, (2): A1 sense: 5'-GAAATTAATTTTTTATATTAACCAAAATTGCGC-3' or A2 sense: 5'-GGAACCAAAATTGCTATAAAAATTTAAAAAAAA-3', (3) A1 antisense: 5'-GCGCAATTTTGGTTAATATAAAAAATTAATTC-3'or A2 antisense: 5'-

TTTTTTTTAAATTTTTATAGCAATTTTGGTTCC-3', and (4) 5'-CCAGATCTCATTTTTATTTTTTATTTTTTAAT-3', which overlaps the start codon of *rnrB* and introduces a *Bgl*II restriction site. Mutations relative to the wild-type sequence are indicated by the underline. In the first round of PCR, two products were generated that contain the mutation at one end. The PCR conditions were as follows: 1x PCR buffer (20 mM Tris pH 9.5, 25 mM KCl, 0.05% Tween-20, 0.1 mg/ml BSA with 2.5 mM $MgCl_2$), 50 μM dNTP mix, 50 μM dATP, 50 μM dTTP, 50 ng of template, 100 nM each primer, and 5 units of Taq DNA polymerase. Cycling conditions were: denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 1 minute. The template was then removed by digestion with *Dpn*I, a restriction enzyme that only cuts methylated DNA, and therefore that does not digest *in vitro*-generated products. The PCR products were cleaned using Qiaquick PCR purification columns (Qiagen) and used as template for a second round of PCR with primers 1 and 4 using the same conditions as described above.

The PCR products were cleaned with Qiaquick columns and digested with *Xba*I and *Bgl*II. They were then ligated to RnrB-ile-αpgal (MacWilliams *et al.*, 2001) that had also been digested with *Xba*I and *Bgl*II and gel purified with the GeneClean kit (BioCan). The resulting products were sequenced to confirm that the mutation had been introduced. The Δ-450/A1-ile-αpgal and Δ-450/A2-ile-αpgal mutants were sequenced from the 5' end with an oligonucleotide overlapping box D of the following sequence: 5'-TTTCTAGAATCGGAGTGGTACCC-3'. The Δ-280/A1 and Δ-280/A2 constructs were sequenced with an antisense oligonucleotide in the coding region of the reporter gene with the primer: 5'-CTTTGTTGATCTGGAGGGATACC-3'.

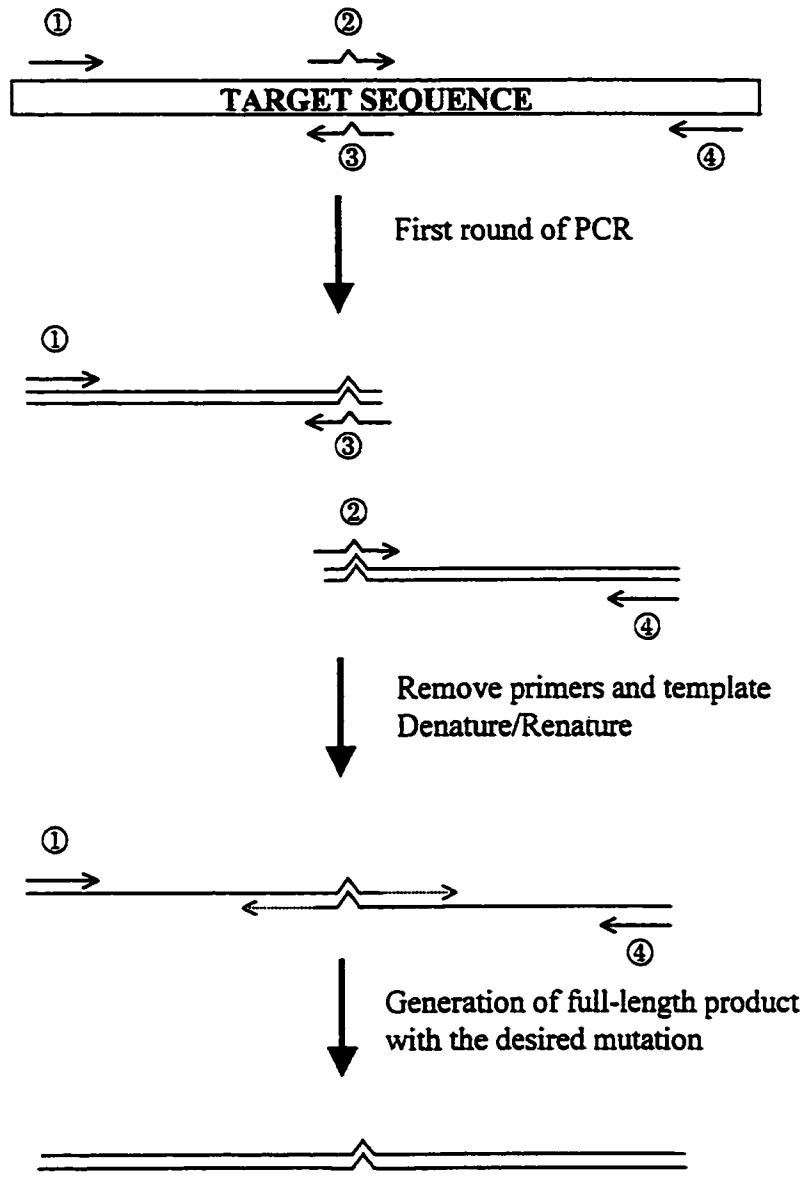


FIGURE 1. Strategy for site-directed mutagenesis of box A of the *rnrB* promoter. The template is amplified in two independent reactions, one using primers 1 and 3, and the second one using primers 2 and 4. The two products are then used as template using primers 1 and 4 for the amplification. This results in a full-length product containing the desired mutation.

2.4. Disruption of the *rnrB* promoter by homologous recombination

The strategy for disruption of the *rnrB* promoter by homologous recombination is depicted in Figure 2. The pRHI100 plasmid (a gift from Robert H. Insall) was digested with *Xba*I and EcoRI to generate a fragment containing the blasticidin resistance gene as well as promoter and terminator sequences. This fragment was cloned into the *Xba*I and *Eco*RI sites of the internal deletion –359Δ-212 (Gaudet and Tsang, 1999; Section 2.2) in such a way that the fragment between –212 and –450 of the *rnrB* promoter was replaced by the blasticidin resistance fragment. The resulting construct was linearized at *Spe*I and *Bam*HI sites and cleaned by phenol: chloroform extraction. The linearized DNA was introduced in *Dictyostelium* by electroporation (Tuxworth *et al.*, 1997) and selected with 10 μg/ml blasticidin S (ICN). Genomic DNA was extracted as described (Nellen *et al.*, 1987) from approximately 50 different clones and analyzed by Southern blot (Sambrook *et al.*, 1989).

2.5. Treatment of *Dictyostelium* cells with drugs and cell survival assays

For treatment of vegetative cells, stock solutions of the drugs were added directly to growing cells in HL5 medium. For treatment with chemical agents during early development, the cells were developed in suspensions of KKP for 4 h prior to the addition of drug solutions. Cells irradiated with UV and cells treated with genotoxic agents during late development were developed on filters saturated with KKP at 10^6 cells/cm^2. For treatments with chemical agents, the filters were placed on pads of blotting paper that had been saturated with KKP containing drugs at the specified concentrations. UV treatments were performed with a UV cross-linker (Stratalinker 1800, Stratagene).

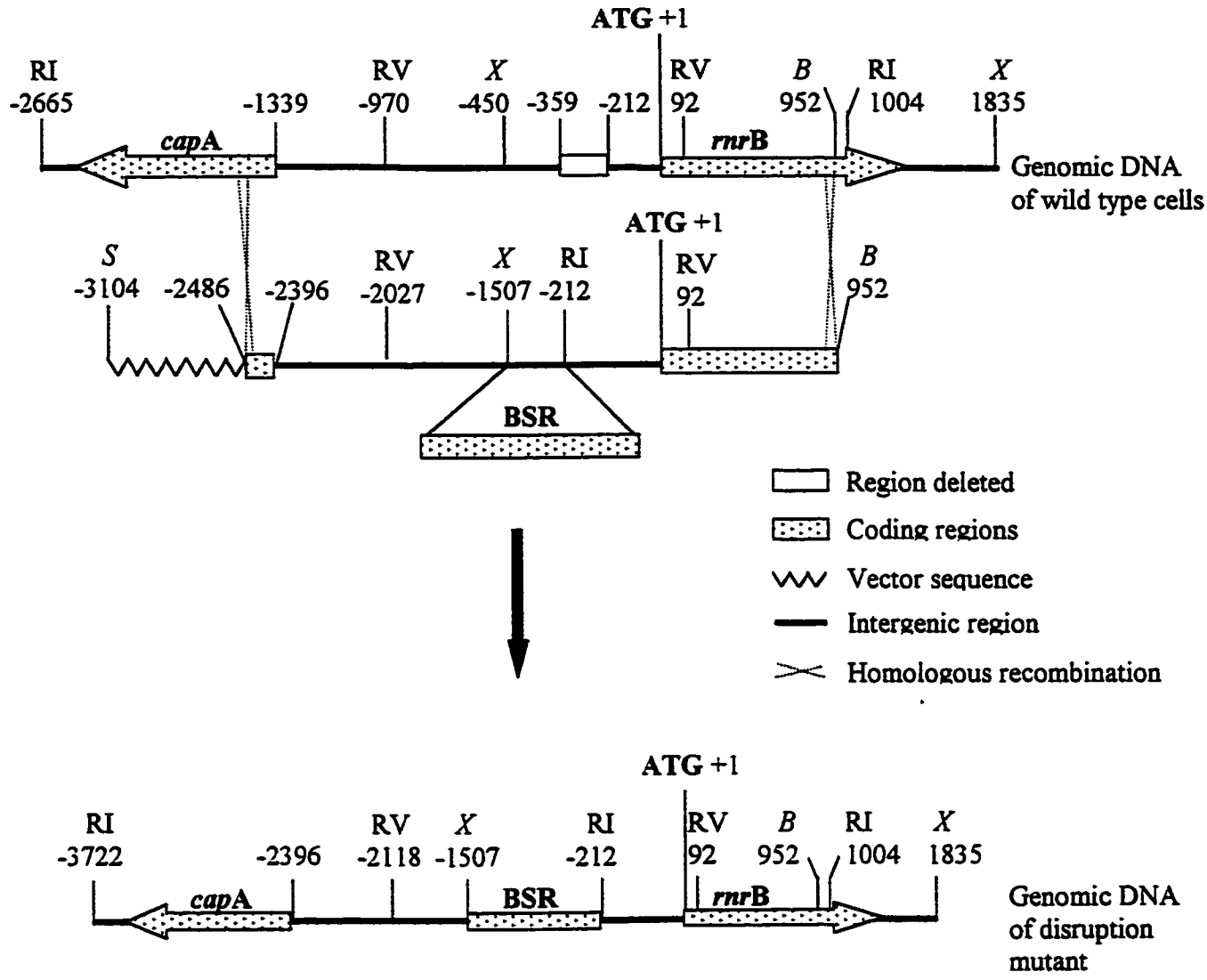


FIGURE 2. Strategy for replacement of the *rnrB* promoter by the blasticidin-resistance gene.

The numbers indicate the distance in bp using the A of the first codon of the *rnrB* coding sequence as a reference, shown as +1. Restriction enzyme sites are abbreviated as follows: *Eco*RI: RI; *Eco*RV: RV, *Bam*HI: *B*; *Xba*I: *X*; *Spe*I: *S*.

Calibration of the UV lamp was verified using uridylic acid as a chemical actinometer (Smith, 1977) correcting for absorption by the solution (Morowitz, 1950). Methyl methane sulfonate (MMS), 4-nitroquinoline-1-oxide (4NQO) and cycloheximide were purchased from Sigma. Hydroxyurea was obtained from ICN.

Following treatment with genotoxic agents, the cells were diluted in KKP buffer. Aliquots of the various dilutions were spread together with *Enterobacter aerogenes* on SM plates. Survivors were scored by counting the number of plaques on the SM plates (Gaudet and Tsang, 1999).

2.6. RNA analyses

Cells were collected by centrifugation and washed once with cold KKP buffer. The cell pellets were frozen on dry ice and kept at -70°C until the RNA was extracted according to Franke *et al.* (1987). Cell pellets containing up to 2 x 10^7 cells were resuspended by vortexing in 200 µl of GSEM buffer (50% guanidine thiocyanate, 0.5% sarkosyl, 25 mM EDTA, 0.1% 2-mercaptoethanol, pH 7.0). One volume of phenol and one volume of chloroform were added. The sample was vortexed vigorously for 1 min and centrifuged for 5 min. The aqueous phase was transferred to a fresh tube. The phenol: chloroform extraction was repeated two more times, and then the nucleic acids were extracted twice with chloform only. The nucleic acids were precipitated with 0.3 M sodium acetate and 2 volumes of 95% ethanol at -70°C, centrifuged for 10 minutes and rinsed with 70% ethanol. The pellets were air-dried and resuspended in DEPC-treated water. The nucleic acids were quantified spectrophotometrically.

For Northern blot analysis, 10 μg of RNA were mixed with ethidium bromide and resolved on formaldehyde gels as described (Fourney *et al.*, 1988). After electrophoresis, the gels were visualized under a UV illuminator to ensure even loading. Nucleic acids were transferred onto Nytran membranes (Schleicher & Schuell) in 10x SSC and cross-linked using a UV cross-linker (Stratalinker 1800, Stratagene). Radioactive probes were generated by random priming following the manufacturer's protocol (Pharmacia). Briefly, 25 ng of DNA were denatured by boiling and chilled on ice. The labelling reaction contained 15 μl of random primers buffer (0.67 M HEPES, 0.17 M Tris-Cl, 17 mM $MgCl_2$, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA containing 18 OD_{260} units hexamers/ml, pH 6.8), 20 μM of each dGTP, dATP and dTTP, 5 μl of [α-^{32}P]dCTP (ICN) (3000 Ci/mmol) and 10 units of Klenow DNA polymerase (MBI Fermentas) in a final volume of 50 μl. The reaction was incubated at room temperature for several hours. Unincorporated nucleotides were removed by passage through a Sephadex G-50 (Pharmacia) size exclusion column. The DNA was denatured again before addition to the prehybridization solution. The *rnrB* probe was the *Eco*RI-*Dra*I fragment of the *rnrB* coding sequence, a region not present in the *rnrB/lacZ* reporter construct used to make the deletions of the *rnrB* promoter (Tsang *et al.*, 1996). Alternatively, for RNA extracted from cells not bearing these constructs, we used a full-length cDNA clone encoding *rnrB* (SSF884) obtained from the University of Tsukuba (Japan) (Morio *et al.*, 1998). Hybridizations were conducted in Denhardt's hybridization solution (6x SSC (0.9 M NaCl, 0.09 Na_3citrate), 5x Denhardts' reagent (0.1% BSA, 0.1 % Ficoll, 0.1% polyvinylpyrrolidone), 0.5% SDS, 100 μg/ml denatured, sonicated herring sperm DNA) containing 50% formamide (Sambrook *et al.*, 1989). Hybridizations and stringency

washes were performed as follows: the blots were hybridized at 40°C overnight and washed twice for 30 minutes in 1x SSC, 0.1% SDS at 65°C; except for the *lacZ* and the *capA* probes, for which hybridization temperature was 45°C and the washes were done in 0.1x SSC, 0.1% SDS at 65°C. Blots were exposed to Kodak X-Omat films with intensifying screens. For each experiment, the same blot was hybridized with different probes. Between each hybridization, the probe was stripped from the membrane by incubating twice for 15 minutes in a boiling solution of 0.1x SSC and 0.5% SDS.

For dot blot analysis, 10 μg of total RNA were treated with 0.3 units of RQ1 RNase-free DNase (Promega) for 30 minutes at 37°C. This suspension was mixed with 3 volumes of denaturation solution (37% formaldehyde, 100% formamide and 20x SSC, in a 7:20:2 ratio), heated at 65°C for 15 minutes, and chilled on ice. Two volumes of 20x SSC were then added to the solution. The RNA samples were spotted in duplicates (5 μg per spot) onto Nytran membranes that had been washed with 10x SSC. The membrane was washed again with 10x SSC and finally the nucleic acids were cross-linked.

To determine the level of expression of the reporter transcript, blots were quantified using a phosphorimager (BioRad GS-363) and the signal intensities were determined using Molecular Analyst™ software (BioRad). The fold-induction of *rnrB* was determined by dividing *rnrB* transcript level in treated cells by that of untreated cells. On average, induction for 25 mM MMS and 10 μg/ml 4NQO was 7.5-fold and 15-fold, respectively. To compensate for variations among experiments, a correction factor was used to calculate the fold induction for the reporter gene activity. The correction factor was obtained by dividing the average induction level for *rnrB* by that of the observed induction level. Thus, if the observed induction for 4NQO was 30-fold, the

correction factor would be 15/30 or 0.5. The fold-induction of the reporter transcript was calculated by dividing the level of *lacZ* message in treated cells by that of the untreated cells, then multiplying this value by the correction factor (Gaudet and Tsang, 1999).

2.7. Assay for β-galactosidase

β-galactosidase activity was assayed using Galacton-Light Plus™, a chemiluminescent substrate (Tropix). Cells were harvested in KKP buffer, pH 8.0 and lyzed by freezing. Cells were thawed in 100 mM $NaPO_4$ containing 1 mM DTT and centrifuged for 10 min at 4°C to remove membranes. The supernatant was transferred into a fresh tube, and protein concentration was determined using Bradford assay (Bradford, 1976). The protein was diluted to 500 µg/ml. The substrate, Galacton-Light Plus, was diluted 1:100 and 10 µl of sample were added to 60 µl of substrate. After a reaction time of 30 min, 100 µl of Light Emission Accelerator were added to each sample and the chemiluminescent product was detected with a Berthold Lumat LB9501 luminometer (MacWilliams *et al.*, 2001).

2.8. Histological stainings

Cells were grown on bacteria and developed on pre-boiled nitrocellulose filters resting on KKP-saturated pads at a density of about 2×10^6 cells/cm^2. At the slug stage, the filters supporting the slugs were fixed in 0.1% glutaraldehyde in Z buffer (containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4 and 1 mM $MgSO_4$, pH 7.0) for 10 min and assayed for ß-galactosidase activity by incubating in Dingermann's cocktail (5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 1 mM EGTA and 1 mM X-gal in Z-buffer) until staining was

observable under a light microscope (Dingermann *et al.*, 1989). The reaction was stopped with 3% TCA. The filters were washed in water, mounted on microscopic slides under coverslips, and examined on a Zeiss Axiophot microscope with a 10X objective. Pictures were taken on Kodak Royal Gold ASA 25 film (Bonfils *et al.*, 1999).

2.9. Electrophoretic mobility shift assays (EMSA)

The cytosolic and nuclear extracts were prepared as described (Schnitzler *et al.*, 1994). Cells were resuspended in lysis buffer (50 mM Tris-Cl pH 7.5, 40 mM $MgCl_2$, 20 mM KCl, 2 mM DTT, 5% sucrose, 0.15 mM spermine, 0.15 mM spermidine and 10% percoll) containing protease inhibitors (0.1 mg/ml phenylmethyl sulfonyl fluoride, 10 µg/ml antipain, 1 µg/ml pepstatin A, 0.1 mg/ml benzamidine) and lysed by passing through a 5 µM polycarbonate filter. The suspension was centrifuged, and the supernatant fraction was saved as the cytoplasmic fraction and dialyzed in 4x binding buffer (20 mM Tris pH 8.4, 240 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.05% Triton X-100). The nuclei were resuspended in 25 mM Tris pH 7.5, 12.5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 20% glycerol and 0.35 M $(NH_4)_2SO_4$, and nuclear proteins were extracted by incubating at 4°C with gentle rocking. The salt-extracted material was cleared by centrifugation at 100,000g. The supernatant was concentrated by a 60% $(NH_4)_2SO_4$ precipitation, followed by dialysis in 4x binding buffer (see above).

The probes were oligonucleotides reconstituting boxes A and B of the *rnrB* promoter as well as the CAE-1 from the *SP60* promoter (Haberstroh *et al.*, 1991). Unlabelled oligonucleotides corresponding to these sequences along with box C and box D, as well as two mutant forms of box A, named box A1 and box A2, were used in

competition assays. The sequences of all the synthetic oligonucleotides used in EMSA are shown in Table 1. Box A1 and box A2 are the same oligonucleotides as those used for site-directed mutagenesis (Section 2.3). The oligonucleotides were quantified spectrophotometrically, and equimolar ratios of sense and antisense strands were annealed in 10 mM Tris and 200 mM NaCl by boiling the solution and letting it cool down at room temperature. Ten units of polynucleotide kinase (MBI Fermentas) were used to end-label 0.1 pmol of oligonucleotide in 1x forward buffer (70 mM Tris-Cl pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol) containing 5 µl of [γ-^{32}P]ATP (3000 Ci/mmol). The reaction was carried out at 37°C for 30 min and stopped by adding EDTA to 10 mM. The unincorporated nucleotides were removed by passage through a Sephadex G-50 column.

For the binding assays, 10 µg of protein extract were incubated with 3000 cpm of end-labelled probe (0.1 to 0.5 ng), 500 ng of double-stranded poly[dI-dC] (Sigma), and 1 µg of BSA in a final volume of 20 µl of 1x binding buffer (see above). The components were allowed to bind at room temperature for 30 minutes, immediately applied on a 4.5% acrylamide-TBE gel and resolved at 4°C at 140V until the unbound probe reached the bottom of the gel. Following electrophoresis, the gel was fixed in 10% acetic acid, dried, and exposed to X-ray films (Kodak X-Omat) (Bonfils *et al.*, 1999).

TABLE 1. Sequence of the synthetic oligonucleotides used for EMSAs

Box A (sense)	5'-GATCCATAGGAACCAAAATTGCGCTAA-3'
Box A (antisense)	5'-GATCTTAGCGCAATTTTGGTTCCTATG-3'
Box B (sense)	5'-GATCCTTTCGGGAATGTGTGTGTATTA-3'
Box B (antisense)	5'-GATCTAATACACACACATTCCCGAAAG-3'
Box C (sense)	5'-GATCCATTGGAACGGTGATTCCATCAA-3'
Box C (antisense)	5'-GATCTTGATGGAATCACCGTTCCAATG-3'
Box D (sense)	5'-GATCCTCTAGAATCGGAGTGGTACCCAAAA-3'
Box D (antisense)	5'-GATCTTTTGGGTACCACTCCGATTCTAGAG-3'
Box A-M1 (sense)	5'-GAAATTAATTTTTTATATTAACCAAAATTGCGC-3'
Box A-M1 (antisense)	5'-GCGCAATTTTGGTTAATATAAAAAATTAATTC-3'
Box A-M2 (sense)	5'-GGAACCAAAATTGCTATAAAAATTTAAAAAAAA-3'
Box A-M2 (antisense)	5'-TTTTTTTTAAATTTTTATAGCAATTTTGGTTCC-3'
CAE-1 (sense)	5'-GATCTTTTTCACACACCCACACACTAATTTACCCCATTTTTG-3'
CAE-1 (antisense)	5'-GATCCAAAAATGGGGTAAATTAGTGTGTGGGTGTGTGAAAAA-3'

3. RESULTS

3.1. Defining the *rnrB* promoter

Similar to other *Dictyostelium* promoters, the 5' untranscribed region of the *rnrB* gene contains over 85% A and T residues with clusters of G/C-rich sequences of approximately 15-20 bp in length. The sequence of the 450 bp upstream of the open reading frame making up the *rnrB* promoter is shown in Figure 3 (Tsang *et al.*, 1996). In several *Dictyostelium* promoters that have been analyzed previously, G/C boxes have been shown to be important for control of gene expression (Haberstroh *et al.*, 1991; Pears and Williams, 1987). This 450-bp fragment of the *rnrB* gene is sufficient to confer regulation on reporter genes during the cell cycle, in response to DNA damaging agents and during multicellular development. The positions of the four G/C-rich boxes contained in the *rnrB* promoter are indicated in Figure 3. We refer to these sequences as boxes A, B, C and D, from the most proximal to the transcription start site to the most distal, respectively (Bonfils *et al.*, 1999).

3.2. Expression of the *rnrB* gene is cell cycle-regulated

In all species studied until now, the genes coding for ribonucleotide reductase are regulated during the cell cycle. They normally begin expression at the G1/S transition point. Since the *Dictyostelium* cell cycle lacks a detectable G1 phase it is difficult to predict how *rnrB* is regulated during the cell cycle. In collaboration with Dr. Harry MacWilliams (MacWilliams *et al.*, 2001), we have examined the cell cycle regulation of the *rnrB* gene using AX2 cells transformed with a plasmid, RnrB-ile-αpgal, that contains the 450-bp *rnrB* promoter fused to an unstable version of β-galactosidase.

A.

box D

-450 TCTAGAATCG GAGTGGTACC CAAATATAGC TTTTTTTTTT TTTTTTGAAA

box C

-400 AATTATTAAA ATTTAATTTA TTGGAACGGT GATTCCATCA TAAACAAAAA

box B

-350 CAAATAAAAT AAATAAAATT TTAATATTTT TTTCTTTCGG GAATGTGTGT

-300 GTATTTTCCA AAAAAAAAAA AAATATTCTG TTTTTTTTAA AAAAAAGAAA

box A

-250 TTAATTTTTT ATAGGAACCA AAATTGCGCT AAAAATTTAA AAAAAAATAA

-200 AAAATTCAAA TTTTTTTTTT TTTTTTTTTT ACTTTTTTTT TTTTTTTATT

-150 ATTATTTATA ACAAATAAAT ATTATTTTTT AATTTTATTA TTCATATTAT

-100 ATAATTTTGG ATTGATTTCA AAACTTAATA AAATCTCATT GTACATTAAA

-50 TTTTATAAAA TAAAATAAAA AAAAAAAAAA TTAAAAAATA AAAAATAAAA

+1 ATG

B.

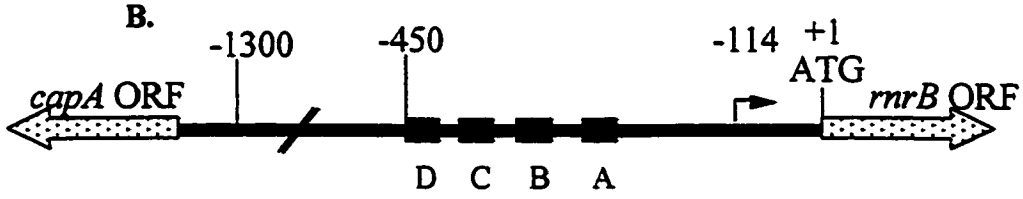


FIGURE 3. 5' upstream region of *rnrB*.

Panel A shows the sequence of the 450 bp upstream of the start codon of *rnrB*. The boxed sequences show the G/C-rich regions referred to as boxes A, B, C, and D in the text. A schematic representation of the *rnrB* gene is depicted in panel B, including the position of boxes A, B, C and D, as well as the position of the gene flanking *rnrB*, *capA*. The transcription start site is indicated by an arrow.

The cells were synchronized by the cold shock method in which cells are maintained at low temperature (9.5°C) for 14-16 h, then warmed rapidly to room temperature. Samples were taken every hour for 9 hours, the approximate duration of one cell cycle. The fraction of cells in S-phase was determined by bromodeoxyuridine (BrdU) labelling, and samples were taken for measurement of β-galactosidase activity and for RNA extraction. After size separation by formaldehyde gel electrophoresis and blotting, the RNA was probed for both the endogenous *rnrB* message and the *lacZ* message. The levels of transcripts were quantified with a Phosphorimager and loading was corrected by dividing by the level of *capA* transcript, which in these experiments corresponded closely to that of the ribosomal RNA (data not shown) (MacWilliams *et al.*, 2001).

The BrdU incorporation curve and the β-galactosidase activity peaked 4 h after release from the cold shock (Figure 4A). Both the endogenous *rnrB* message and the reporter message were highest from 2.5 to 3 h. In *Dictyostelium*, mitosis lasts about 15 minutes and the S phase has been estimated at 30 minutes (Weijer *et al.*, 1984). Since G1 is undetectable, and the rest of the cell cycle is G2, the interval from the beginning of M until the midpoint of S is about 30 minutes. The approximate 1-h delay between the *rnrB* message peak and the S phase maximum thus places *rnrB* message peak in late G2. Similar results were obtained with untransformed cells (data not shown).

We wished to confirm the cell-cycle dependent expression of *rnrB* using an independent method. Cells were thus synchronized by the high density method in which cells are allowed to go into stationary phase. These cells stop dividing, and when transferred to fresh medium they start dividing synchronously. Samples were taken at 1-h intervals over a period of 12 h for measurement of *rnrB* promoter activity and BrdU

incorporation. Since the RnrB-iαgal reporter levels accurately reflected the *rnrB* message (see Figure 4A) and is considerably easier to assay, we used the reporter to assay promoter activity. The results of this experiment are shown in Figure 4B. The fraction of cells in S phase peaked 4 h after release from high density, as we had seen for the cold shock. After high-density synchronization, however, the *rnrB* promoter activity was maximal about 3 h after the S phase (Figure 4B). This was seen in most individual experiments as well as in the overall average. These results suggest that there are two peaks of *rnrB* expression during the *Dictyostelium* cell cycle. This is supported by other experiments done with unsynchronized cells (see Discussion; MacWilliams *et al.*, 2001).

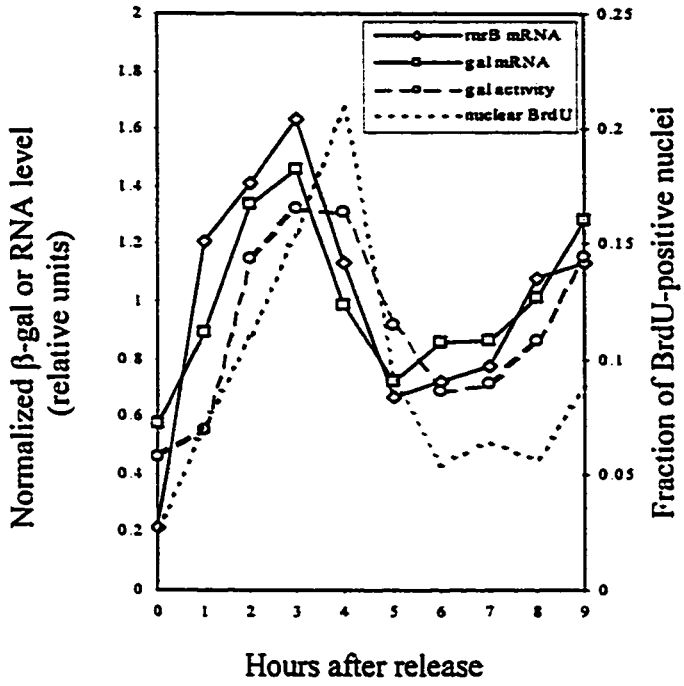
3.2.1. Role of box A in regulating the cell-cycle expression of *rnrB*

Since box A bears homology to the MluI box involved in cell cycle control of gene expression in *S. cerevisiae* (reviewed by Andrews and Herskowitz in 1990), we examined the ability of a truncated *rnrB* promoter, containing only 280 bp upstream from the ATG site and including box A, to direct cell-cycle regulation on the ile-αpgal reporter gene. Cells were cold synchronized and assayed for β-galactosidase activity. Figure 5 shows that the Δ-280-ile-αpgal construct exhibits cell-cycle-regulated activity, similar to that of the entire *rnrB* promoter (Figure 4). Two mutations have been inserted in the Δ-280 construct, A1 and A2, resulting in Δ-280/A1-ile-αpgal and Δ-280/A2-ile-αpgal (see Figure 5). Cells transformed with these constructs were tested for expression of β-galactosidase during the cell cycle (Figure 5).

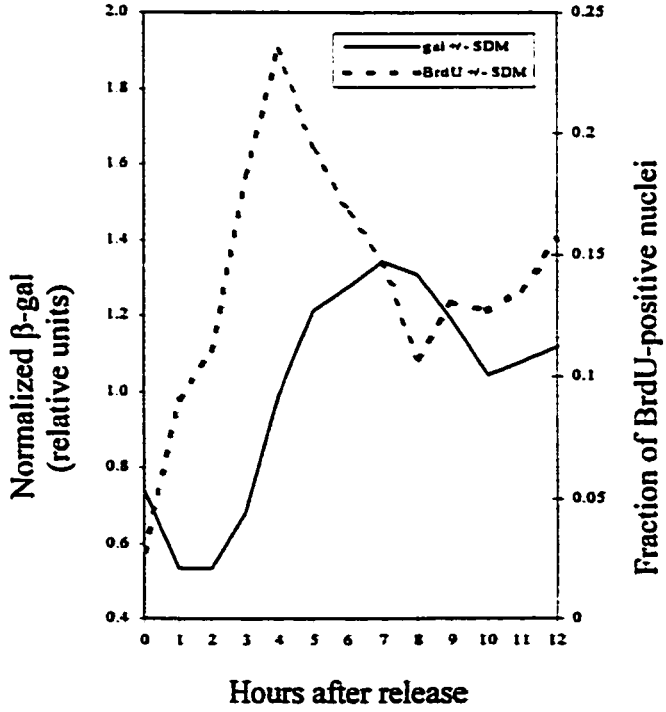
FIGURE 4. Cell cycle regulation of *rnrB* after synchronization.

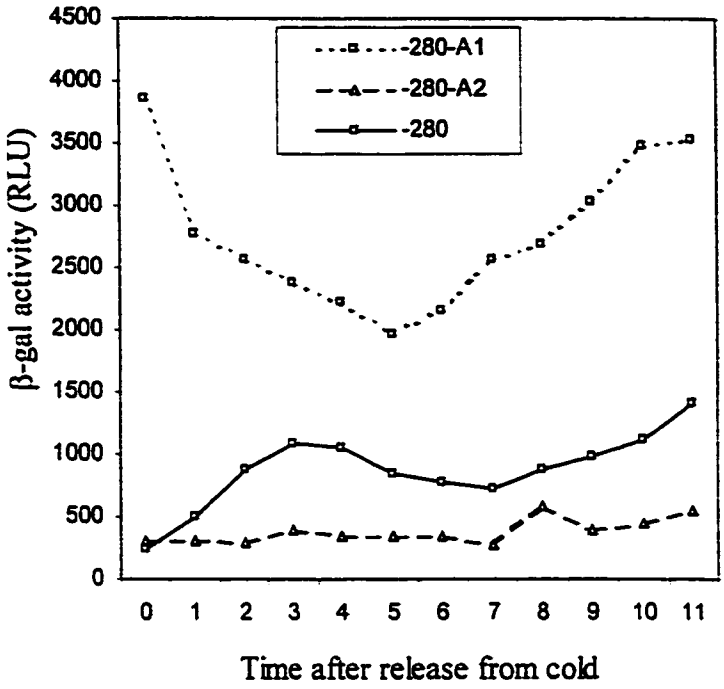
AX2 cells transformed with the RnrB-ile-αpgal plasmid were synchronized. Samples were taken every hour to assay the proportion of S phase cells as indicated by the number of BrdU labelled cells, the β-galactosidase activity directed by the *rnrB* promoter, as well as the levels of *lacZ* and endogenous *rnrB* transcripts. Cells were synchronized by the cold shock method (panel A) or by the high density method (panel B).

A. Cold synchronized cells



B. High density synchronized cells





WT box A: 5'-ATAGGAACCAAAATTGCGCTT-'3

box A1: 5'-ATATTAACCAAAATTGCGCTT-'3

box A2: 5'-ATAGGAACCAAAATTGCTATT-'3

FIGURE 5. Cell-cycle regulated expression directed from wild-type and mutated versions of box A.

Transformants bearing the Δ-280-ile-αpgal, the Δ-280/A1-ile-αpgal or the Δ-280/A2-ile-αpgal constructs were synchronized by cold shock. Samples were collected every hour for 11 hours and assayed for β-galactosidase activity.

The Δ-280/A1-ile-αpgal construct has 3-10-fold higher expression than the wild type construct, suggesting that the A1 site is probably a transcriptional repressor element. The Δ-280/A1-ile-αpgal construct also showed periodic expression during the cell cycle; however the expression profile was very different from that directed by the wild type promoter.

The Δ-280/A2-ile-αpgal construct displayed very low levels of expression, indicating that the A2 site may be a transcriptional activating sequence.

3.3. Response of the *rnrB* gene to DNA-damaging agents

The genes encoding ribonucleotide reductase, as well as many other genes activated by DNA-damaging agents are also under cell-cycle control. Therefore, analysis of the effects of DNA-damaging agents in proliferating cells may be complicated by mechanisms overlapping the repair and growth processes. The life cycle of *Dictyostelium* consists of two mutually exclusive phases: the growth phase and the developmental phase. During *Dictyostelium* development the expression of genes and proteins involved in growth are down-regulated. Our laboratory has previously shown that the *rnrB* transcript is present in vegetative cells, nearly undetectable in early developing cells and expressed at higher levels in late development (Tsang *et al.*, 1996). We took advantage of the low level of expression of the genes required for growth during development in *Dictyostelium* to study the DNA damage response without complication from growth processes.

3.3.1. DNA-damaging agents stimulate the accumulation of *rnrB* transcript

We examined the effects of the alkylating agent MMS, the UV-mimetic agent 4NQO, and UV irradiation on the expression of the *rnrB* gene in *Dictyostelium*. As shown in Figure 6, the level of *rnrB* transcript increased when cells were exposed to DNA-damaging agents. The increased accumulation of *rnrB* transcript in response to DNA-damaging agents was dose-dependent. The levels of induction elicited by a 1 h treatment with 10 μg/ml of 4NQO, 15 mM of MMS and 30 J/m^2 UV-irradiation were approximately 15-fold, 7.5-fold and 15-fold, respectively. The survival rates for 5 mM, 10 mM, 15 mM and 20 mM of MMS were 99%, 90%, 60% and 30%, respectively. In the case of 4NQO the survival rates for 1 μg/ml, 5 μg/ml, and 10 μg/ml were 99%, 50% and 2%, respectively. Over 95% of the cells survived a dose of 100 J/m^2 of UV irradiation. These values are consistent with the data published previously (Bronner *et al.*, 1992; Payez *et al.*, 1972).

To ensure that the induction of *rnrB* by DNA-damaging agents is a specific event, we investigated the accumulation of *capA* transcripts (Grant *et al.*, 1990). The *capA* gene is located upstream of *rnrB* and is transcribed in the opposite orientation in relation to *rnrB* (Tsang *et al.*, 1996). Unlike *rnrB*, the *capA* gene is constitutively expressed during growth and throughout development (Grant *et al.*, 1990). Figure 6 shows that the levels of the two *capA* transcripts, generated by alternative splicing of a retained intron (Grant *et al.*, 1990), remained relatively unchanged in the presence of various DNA-damaging agents. Differences observed in *capA* transcript level corresponded to variations in the amount of RNA loaded on the gels. The expression of the gene encoding calmodulin was also not affected by DNA-damaging agents (data not shown) (Gaudet and Tsang, 1999).

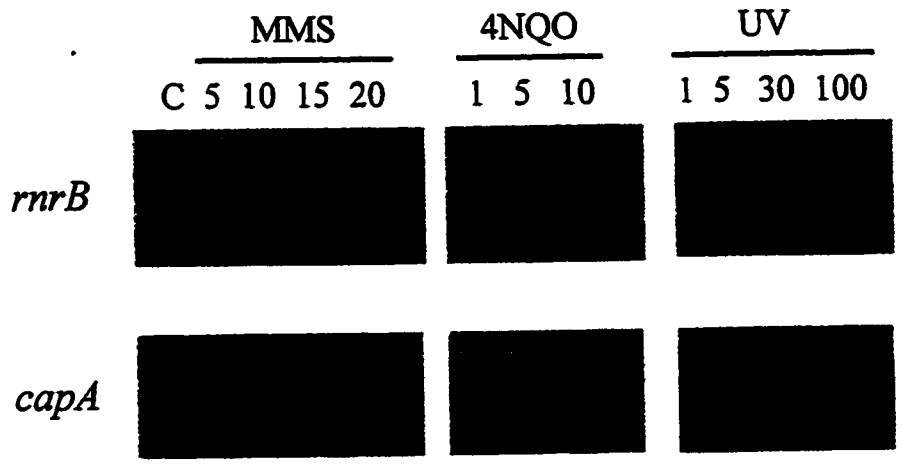


FIGURE 6. Effect of DNA-damaging agents on the accumulation of the *rnrB* transcript.

AX2 cells were developed for 4 h and exposed for 1 h to 4NQO (1, 5 or 10 μg/ml) or to MMS (5, 10, 15 or 20 mM). For UV treatment, 4 h cells were irradiated at 1, 5, 30 or 100 J/m^2, then incubated on pads saturated with KKP for 1 h. Total cellular RNA was extracted, resolved by denaturing gel electrophoresis, and blotted onto membranes. Autoradiographs obtained from probing the same membrane for the *rnrB* gene and the *capA* gene are shown. C represents the untreated control.

3.3.2. Hydroxyurea causes up-regulation of *rnrB* expression in vegetative cells

We tested the effect of the ribonucleotide reductase inhibitor hydroxyurea on the level of *rnrB* transcript in actively dividing (vegetative) and non-dividing (early developing) cells. Figure 7 shows that the presence of hydroxyurea increases the level of *rnrB* transcript. The maximal induction was observed upon treatment of vegetative cells with 5 mM hydroxyurea, and was 5-fold relative to the level of the untreated control. Hydroxyurea had little or no effect on the accumulation of *rnrB* transcript in early development.

The expression of the *capA* gene was not affected by these treatments. The doses we used, ranging from 1 to 100 mM, are known to have dramatic effects on the growth of *Dictyostelium* cells (Deering and Michrina, 1982).

3.3.3. Effect of DNA-damaging agents on *rnrB* expression is independent of developmental stage

The level of *rnrB* transcript is moderate in growing cells, low in the first 10 h of development and high during late development (Tsang *et al.*, 1996). To evaluate the process of DNA damage induction under different physiological conditions we treated vegetatively growing cells, 4 h- and 15 h-developing cells with 4NQO. Figure 8 shows that at these three stages of the life cycle, the cells were capable of responding to 4NQO in the induction of *rnrB*. A similar effect was observed when the cells were treated with MMS or irradiated with UV (see Figure 10). Moreover, the level of induction was very similar for vegetative cells and for cells from different stages of development. At 10 μg/ml of 4NQO, the level of induction was about 15-fold for all stages of the life cycle.

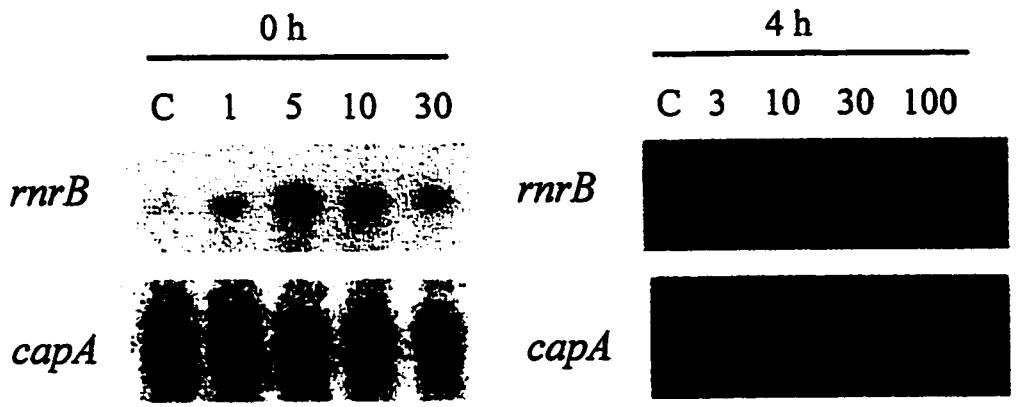


FIGURE 7. Effect of hydroxyurea on the accumulation of the *rnrB* transcript. AX2 cells, either vegetatively growing or developed for 4 h, were exposed for 1 h to hydroxyurea (1, 5, 10 or 30 mM for vegetative cells and 3, 10, 30 or 100 mM for early developing cells). Total cellular RNA was extracted, resolved by denaturing gel electrophoresis, and blotted onto membranes. Autoradiographs obtained from probing the same membrane for the *rnrB* gene and the *capA* gene are shown. C represents the untreated control.

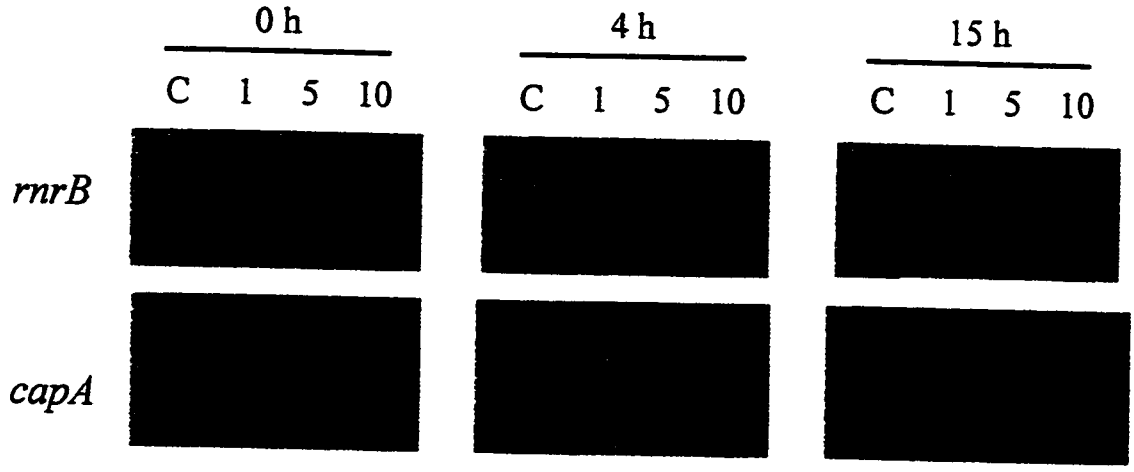


FIGURE 8. Regulation of *rnrB* by DNA-damaging agents during growth and development.

Growing cells and cells that had been developed for different times were treated with 1, 5, or 10 μg/ml of 4NQO for 1 h at the times indicated. Total RNA was extracted and analyzed by Northern blot. Autoradiographs of the same membrane probed with *rnrB* and *capA* are shown. C, untreated control.

We also examined the expression of the *capA* gene and found that the levels of these transcripts varied only slightly. The variations observed did not correlate with either the drug or the dosage used (Gaudet and Tsang, 1999).

3.3.4. Up-regulation of *rnrB* is independent of protein synthesis

We investigated the induction process in the presence of the protein synthesis inhibitor cycloheximide to determine whether the up-regulation of the *rnrB* message by DNA-damaging agents involves pre-existing factors or requires *de novo* synthesis of proteins. Figure 9 shows the effects of 4NQO on gene expression in the presence of cycloheximide. Prior treatment with cycloheximide did not alter appreciably the 4NQO-stimulated increase in accumulation of the *rnrB* transcript. We tested the effectiveness of the cycloheximide treatment by examining the expression of a gene we serendipitously found to be up-regulated by protein synthesis inhibitors. Partial sequence analysis revealed that the clone pB47 encodes a homologue of seryl-tRNA synthase (P. Belhumeur and A. Tsang, unpublished data). Cycloheximide, but not 4NQO, stimulated an increased accumulation of the pB47 transcript. Also shown in Figure 9 are the levels of the *capA* transcripts, which remained relatively unaffected by either of the drug treatments. These results indicate that the induction of *rnrB* by DNA-damaging agents can take place in the presence of a protein synthesis inhibitor (Gaudet and Tsang, 1999).

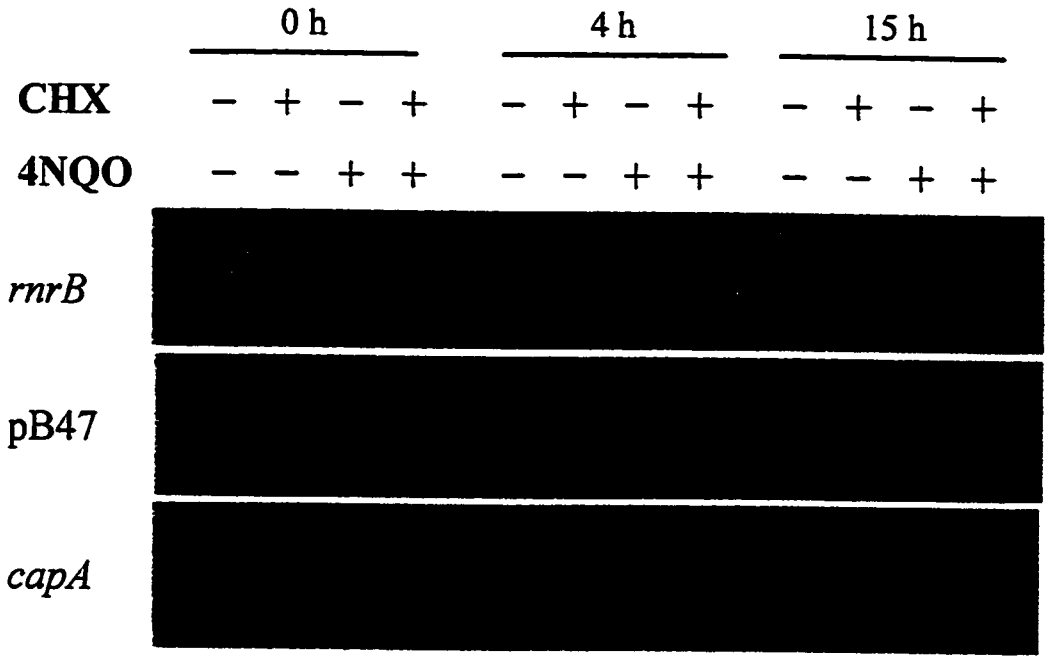


FIGURE 9. Effect of 4NQO and cycloheximide on the accumulation of the *rnrB* transcript.

AX2 cells that had been developed for 4 h were exposed to either 500 μg/ml cycloheximide, 10 μg/ml 4NQO or to both for 1 h. When both drugs were given, cycloheximide was added 10 min before 4NQO. Shown here are autoradiographs of the same membrane probed with *rnrB*, pB47, and *capA*. (+) indicates the presence and (-) shows the absence of the drugs.

3.3.5. Conferring DNA-damage inducibility on heterologous genes

To test whether the 5' upstream region of the *rnrB* gene is sufficient to confer DNA-damage responsiveness, we have made reporter constructs in which the *rnrB* coding region, together with the introns, is entirely absent. These constructs consist of a 450-bp *rnrB* promoter fragment fused to genes encoding GFP, RnrB-ubi-S65TGFP, and a labile version of β-galactosidase, RnrB-ile-αpgal (MacWilliams *et al.*, 2001). Figure 10 shows the levels of the *rnrB* and GFP transcripts expressed by transformed cells bearing the RnrB-ubi-S65TGFP fusion construct following treatment with 10 or 20 mM MMS, 5 or 10 μg/ml 4NQO, or with 30 or 100 J/m^2 UV irradiation. The level of GFP transcript increased in the presence of DNA-damaging agents. The response of the RnrB-ubi-S65TGFP fusion gene appears similar to that of the endogenous *rnrB* gene in all respects. We observed induction of the reporter gene at all stages of the life cycle tested: vegetatively growing cells as well as cells developed for 4 h or 15 h. Similar to that of the endogenous *rnrB* gene, the response was dose-dependent. Irradiation with UV produced a much weaker response in late developing cells, presumably due to the failure of UV light to penetrate through the slime sheath (Gaudet et *al.*, 2001).

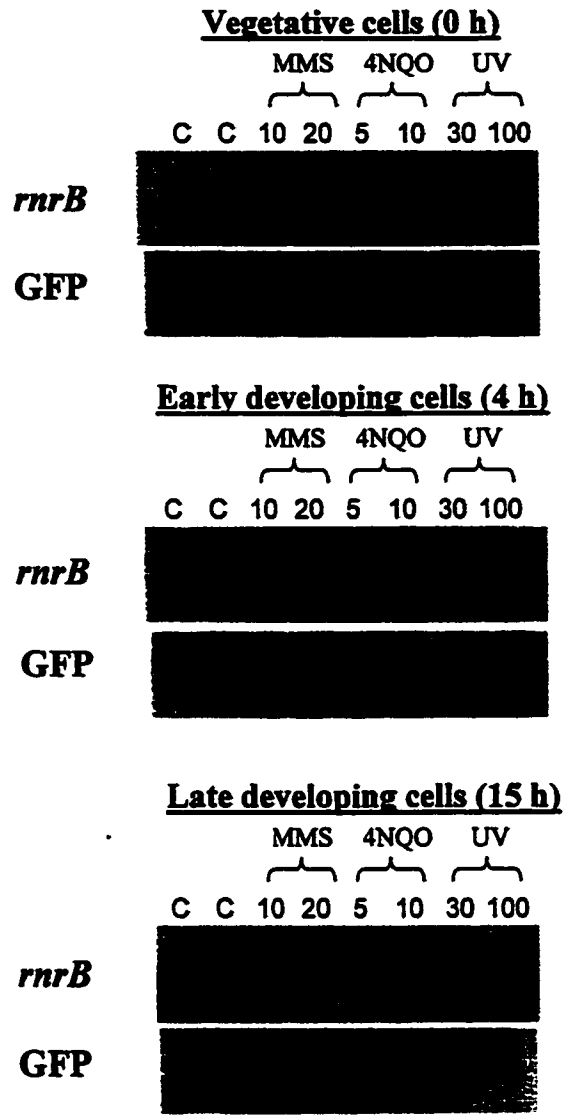


FIGURE 10. Effect of DNA-damaging agents on the accumulation of the *rnrB* and GFP transcripts during growth and development.

AX2 cells transformed with the RnrB-ubi-S65TGFP construct were treated during vegetative growth (0 h) or developed for 4 h or 15 h before treatment. Cells were exposed for 1 h to 10 or 20 mM MMS, or 5 or 10 μg/ml 4NQO. For UV irradiation, cells were treated with 30 or 100 J/m^2 and incubated for 1 h before a sample was collected. Shown here are autoradiographs of blots probed for GFP and *rnrB* transcripts. C indicates the untreated control.

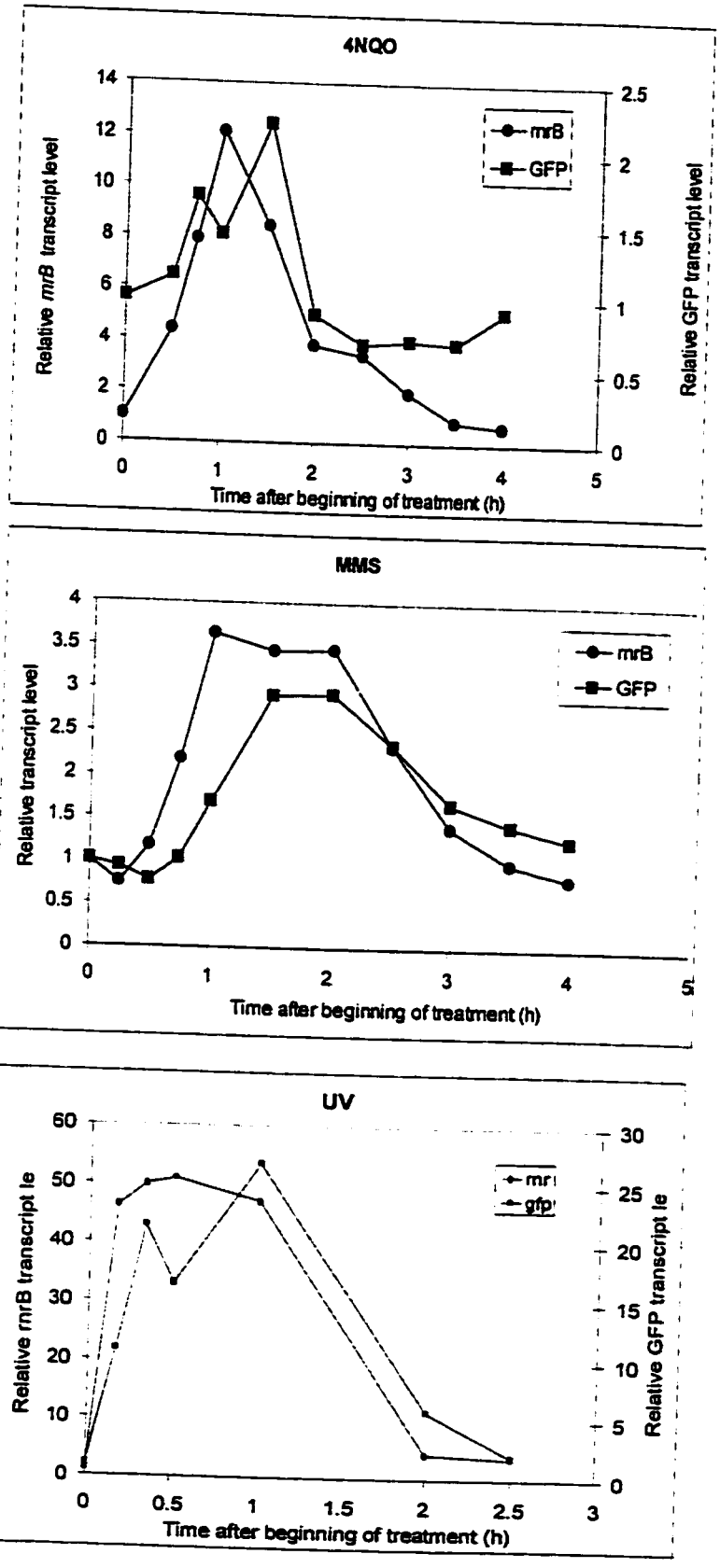
3.3.6. Time course of the DNA-damage response

We have determined the rate of accumulation of the *rnrB* and the GFP transcripts in response to DNA-damaging agents. Figure 11 shows the accumulation of the *rnrB* and the GFP transcripts in the continuous presence of 5 μg/ml 4NQO, 10 mM MMS or 30 J/m^2 of UV light. Response to the different drugs was rapid, an increase in *rnrB* and GFP transcript levels being observable between 10 and 45 minutes after the beginning of treatments. The response of the GFP transcript was similar to that of the endogenous *rnrB* gene, except that the magnitude of the response appeared to be lower than for the endogenous *rnrB* gene. This may be due to the higher basal expression levels of the GFP gene directed by the multicopy plasmid compared to the low basal expression level of the *rnrB* gene. Another possibility is that the transcription factors required for the DNA-damage response are limiting due to the large number of copies of the *rnrB* promoter in these transformants.

3.3.7. *rnrB*-driven gene induction results in up-regulation at the protein level

One important consideration concerning inducible gene expression is whether the gene product is also synthesized, and not only the messenger RNA. Until now, our attempts to produce antibodies against the ribonucleotide reductase small subunit protein have been unsuccessful. To address the question of production of functional gene products, we have studied the production of reporter proteins. We first investigated whether the increased GFP transcript level resulted in an increased level of the GFP protein. Despite the striking induction at the transcript level (see Figures 10 and 11), no large changes in GFP fluorescence were seen. We attribute this to the relative stability of

FIGURE 11. Time course of DNA-damage induction of the *rnrB* and GFP genes. AX2 cells transformed with the RnrB-ubi-S65TGFP construct were developed for 4 h and treated with 5 μg/ml 4NQO, 10 mM MMS or 30 J/m^2 of UV light. Samples were taken periodically, cells were pelleted and frozen until RNA extraction was performed. The RNA was run on denaturing agarose gels, transferred to Nylon membranes and probed sequentially for *rnrB*, GFP, and *capA*. The blots were quantified with a PhosphorImager. To correct for loading, the relative transcript levels of the *rnrB* and GFP genes were normalized by dividing by the level of the *capA* transcript. Values are expressed relative to that of the untreated control.


4NQO
Relative mrB transcript level
Relative GFP transcript level
mrB
GFP
Time after beginning of treatment (h)
MMS
Relative transcript level
mrB
GFP
Time after beginning of treatment (h)
UV
Relative mrB transcript le
Relative GFP transcript le
mr
gfp
Time after beginning of treatment (h)

the GFP protein. In particular, ubi-S65TGFP has been reported to have a half-life of 7 h in early developing cells (Deichsel *et al.*, 1999). GFP can thus accumulate over time, so a significant level of fluorescence will be present in uninduced cells as a result of the basal promoter activity. The amount produced during the short induction period may not result in a large relative increase. To address this problem, we used an unstable β-galactosidase reporter with a half-life of about 30 min, which is designated ile-αpgal. Using this RnrB-ile-αpgal fusion construct we have been able to detect increases in β-galactosidase activity upon treatment with 4NQO, MMS and UV (Figure 12). These results show that the *lacZ* induction by DNA-damaging agents results in the production of a functional enzyme.

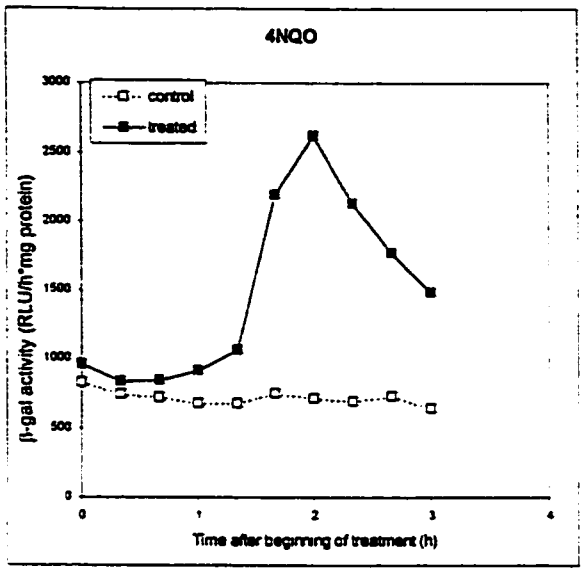
Comparing the results from the β-galactosidase activity with the GFP induction, it appears that the increase in protein level follows that of the transcript level by 30-60 minutes for 4NQO and MMS. UV irradiation gave a different result: no increase in β-galactosidase activity was observable until 3 to 3.5 h after irradiation, which caused a 10-fold increase in GFP transcript level only 10 minutes after irradiation (see Figure 11). This might be due to a partial inhibition of translation after UV-exposure (Kumari and Nair, 1984; Hyodo *et al.*, 1982).

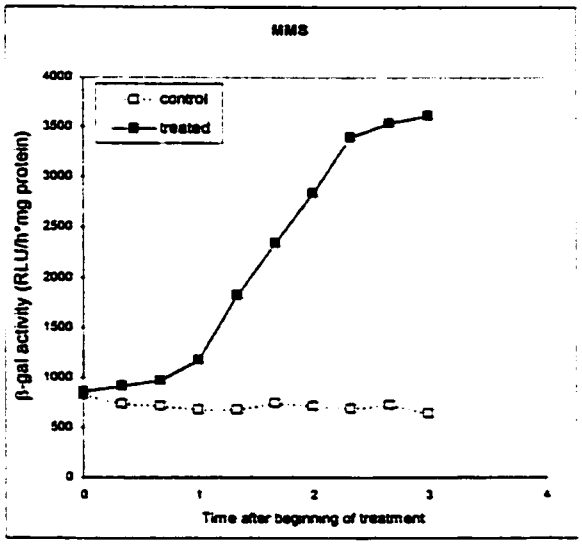
3.3.8. Identification of *cis*-acting elements controlling the DNA damage response

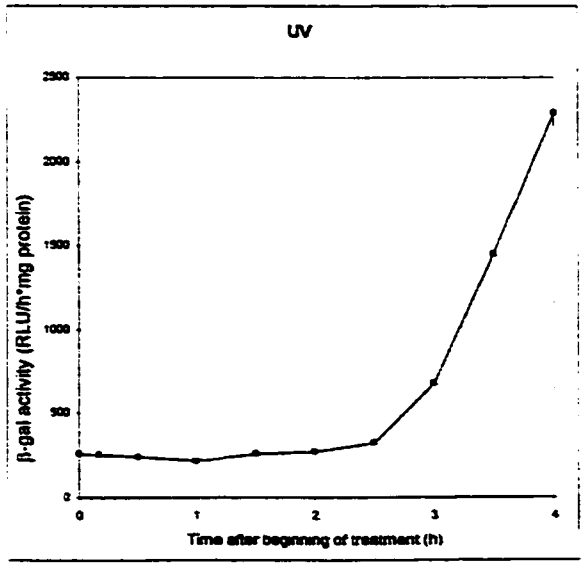
We used promoter deletion analysis to define the *cis*-acting elements of the *rnrB* promoter involved in the response to DNA-damaging agents. A schematic representation of the constructs used in this study is shown in Figure 13. *Dictyostelium* cells carrying the deletion constructs were developed for 4 h and exposed for 1 h to either 10 μg/ml of

FIGURE 12. ß-galactosidase activity of AX2 cells transformed with a RnrB-ile-αpgal fusion construct upon treatment with DNA-damaging agents.

AX2 cells transformed with the RnrB-ile-αpgal construct were developed for 4 h and treated with 5 μg/ml 4NQO, 10 mM MMS or 30 J/m^2 of UV light. Samples were taken periodically and assayed for β-galactosidase activity.


4NQO
control
treated
β-gal activity (RLU/h*mg protein)
Time after beginning of treatment (h)


MMS
control
treated
β-gal activity (RLU/h*mg protein)
Time after beginning of treatment


UV
β-gal activity (RLU/h*mg protein)
Time after beginning of treatment (h)

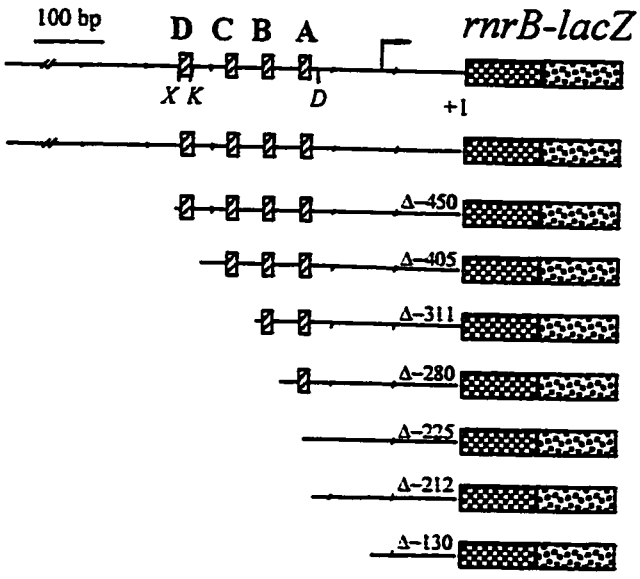
4NQO or 25 mM MMS. The fold-increase in *lacZ* transcript level for each construct in the presence of MMS or 4NQO is shown in Figure 13. Deletion of the upstream region of the *rnrB* promoter up to base -450 did not affect the up-regulation of the gene following treatment with 4NQO or MMS. In addition, cells bearing the internal deletion construct -444Δ-212, missing the four G/C-rich boxes A-D, failed to respond to both drugs. Together these results suggest that the sequence between -444 and -212 contains the elements necessary for the DNA-damage induction of *rnrB*.

A 5' deletion construct missing all the sequence upstream of base -311 rendered the reporter construct unresponsive to 4NQO. Similarly, all constructs missing additional sequence were unable to respond to 4NQO. Therefore in this sequence context, the 94-bp region comprised between -405 and -311 and containing the G/C-rich box C is essential to confer 4NQO-induced DNA damage response. Results from experiments conducted with internal deletion constructs showed that the presence of box D could restore the response to 4NQO (compare constructs -444Δ-212 and -429Δ-212). This suggests that in addition to box C, box D also plays a role in the response to 4NQO. Consistent with the results from the 5' deletions an internal deletion construct bearing box A alone, construct -444Δ-280, was ineffective in promoting 4NQO response on the reporter construct. Construct -444Δ-311, containing both box A and box B, showed a response to 4NQO, which contradicted the results obtained with the 5' deletion constructs. Because this region was unable to confer response to 4NQO in the context of 5' deletion constructs, we concluded that boxes C and D are the major elements involved in the response to 4NQO.

FIGURE 13. Transcriptional response directed by the deletion constructs in the presence of DNA-damaging agents.

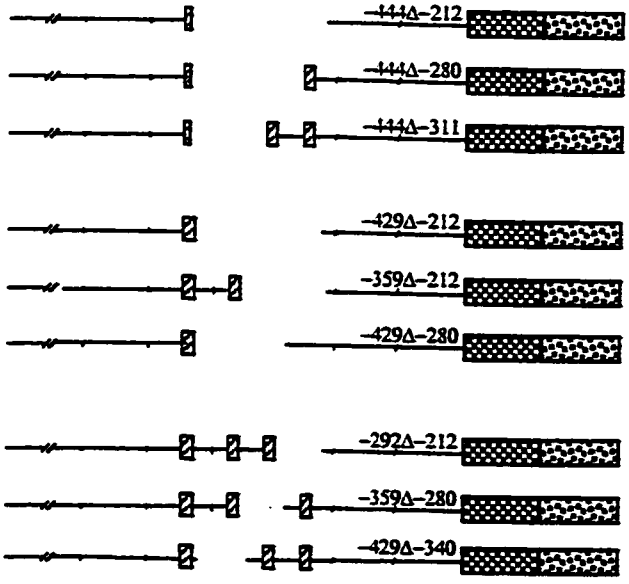
Cells were developed for 4 h and treated with 10 μg/ml of 4NQO or 25 mM of MMS for 1 h. Total RNA was extracted and analyzed by dot blot. The induction values correspond to the increase in the *lacZ* transcript level after correction for the endogenous *rnrB* induction as described in the Materials and Methods section. Each value is the average of two independent experiments ± the standard deviation. A construct was judged damage-inducible (+) if the level of the *lacZ* transcript was at least 2-fold higher than that in untreated cells. Undetectable means that the signal for the *lacZ* transcript was too close to that of the background to be quantified accurately.

5' DELETIONS



	4-NQO		MMS	
	4.0 ± 0.8	+	3.2 ± 0.6	+
Δ-450	4.3 ± 1.4	+	5.5 ± 1.3	+
Δ-405	3.0 ± 0.3	+	2.3 ± 0.3	+
Δ-311	1.0 ± 0.3	-	2.6 ± 0.4	+
Δ-280	1.4 ± 0.1	-	2.0 ± 0.2	+
Δ-225	0.2 ± 0.02	-	0.53 ± 0.01	-
Δ-212	undetectable	-	undetectable	-
Δ-130	undetectable	-	undetectable	-

INTERNAL DELETIONS



	4-NQO		MMS	
-144Δ-212	1.4 ± 0.1	-	1.8 ± 0.8	-
-144Δ-280	1.7 ± 0.9	-	2.3 ± 0.2	+
-144Δ-311	3.1 ± 1.0	+	4.0 ± 1.1	+
-429Δ-212	3.9 ± 0.9	+	1.50 ± 0.03	-
-359Δ-212	2.2 ± 0.3	+	2.8 ± 1.0	+
-429Δ-280	4.9 ± 0.1	+	4.6 ± 1.2	+
-292Δ-212	7.1 ± 3.4	+	3.3 ± 0.3	+
-359Δ-280	12.6 ± 9.8	+	9.2 ± 2.7	+
-429Δ-340	5.2 ± 1.4	+	3.2 ± 2.0	+

When treated with MMS, the transcript encoded by the 5' deletion construct Δ-280 was up-regulated. The only G/C-rich element present in this construct is box A. Constructs with further truncation, Δ-225, Δ-212 and Δ-130, were unresponsive to MMS. Therefore it appears that box A is sufficient for the up-regulation of *rnrB* by MMS. The results obtained using the strains bearing the internal deletion constructs are consistent with this idea. The internal deletion -444Δ-280 (with boxes B-D missing) responded to MMS whereas construct -444Δ-212 (missing boxes A-D) and -429Δ-212 (containing box D alone) did not. However the deletion construct -359Δ-212 containing boxes C and D was inducible by MMS. These results suggest that box C can confer induction by MMS. From these results we conclude that box A and box C are involved in the response to the alkylating agent MMS (Gaudet and Tsang, 1999).

3.3.9. Construction of a mutant defective in the *rnrB* response to mutagens

In eukaryotes, although changes in expression of several DNA-damage-responsive genes have been well documented, few studies have been able to assess the physiological importance of this observation. The mapping of the *cis*-acting elements of the *rnrB* gene responsible for the DNA-damage response (Gaudet and Tsang, 1999) allowed us to test this. We have constructed a plasmid in which boxes A, B, C and D, required for DNA-damage induced expression and developmentally regulated expression of *rnrB*, have been replaced by a selectable marker conferring resistance to blasticidin. The promoter of *rnrB* has been disrupted by homologous recombination as shown in Figure 2. The resulting strain is referred to as RnrB-P-KO (*rnrB* promoter knock-out). We have also isolated clones that were resistant to blasticidin, but in which the *rnrB* locus was not

disrupted. We used these clones as controls to address the possibility that some defects could be caused by the expression of the antibiotic-resistance gene.

The confirmation of the deletion by Southern blotting is shown in Figure 14. The RnrB-P-KO strain retains only the 5' upstream sequence necessary for vegetative expression of the gene, that is, from –212 to the ATG, as we have previously shown (Bonfils *et al.*, 1999).

To confirm that the RnrB-P-KO strain is unable to respond to DNA-damaging agents in the induction of *rnrB*, we have treated RnrB-P-KO cells with mutagens and extracted the RNA as described (Gaudet and Tsang, 1999). As shown in Figure 15, the mutant showed no up-regulation of the *rnrB* transcript following treatment with MMS or 4NQO, while the control strain had a normal response. We have also tested the induction of the gene encoding the large subunit of ribonucleotide reductase, which we call *rnrA*. This gene was responsive to MMS and 4NQO in both the control and the RnrB-P-KO strains.

3.3.10. Physiological effects of a genomic deletion of the DNA-damage response elements

To determine the physiological effect of the absence of *rnrB* transcriptional response to DNA-damaging agents, we first tested the sensitivity of the RnrB-P-KO cells to DNA-damaging agents and compared it to that of the control strain. Figure 16 shows that this deletion causes a 10% decrease in survival to 5-20 mM MMS, and a more dramatic decrease of about 50% for a higher dose (25 mM MMS).

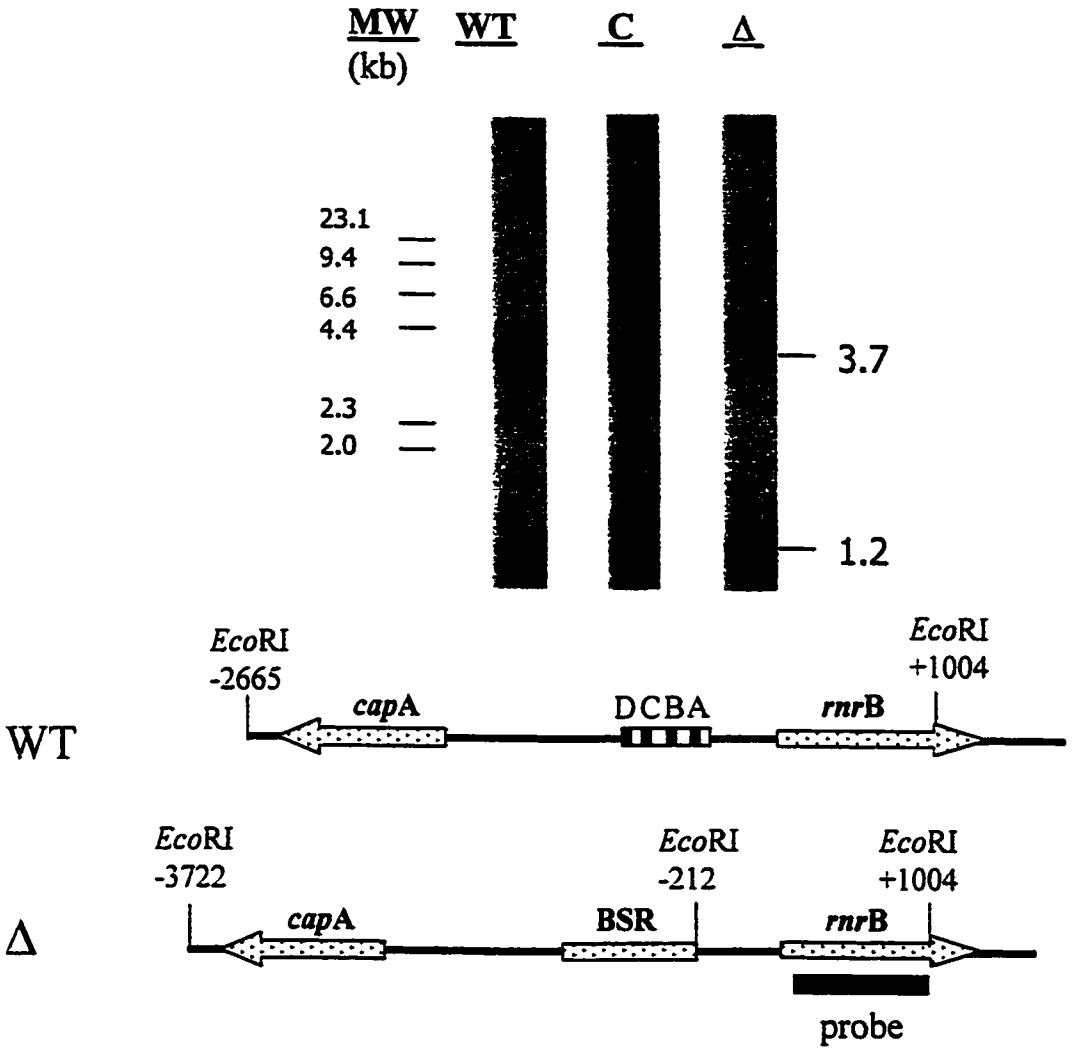


FIGURE 14. Southern blot showing that the *rnrB* promoter locus has been replaced by the BSR marker.

Genomic DNA was extracted from the AX2 parental strain used to construct the mutant (WT), a control transformant (C) and the RnrB-P-KO strain (Δ). The DNA was digested with the enzyme *Eco*RI, and the DNA was resolved on a 1% agarose gel. The DNA was transferred to a nylon membrane and probed using a portion of the *rnrB* coding region as indicated. The size of the restriction fragments produced by the *Eco*RI digestion is also shown.

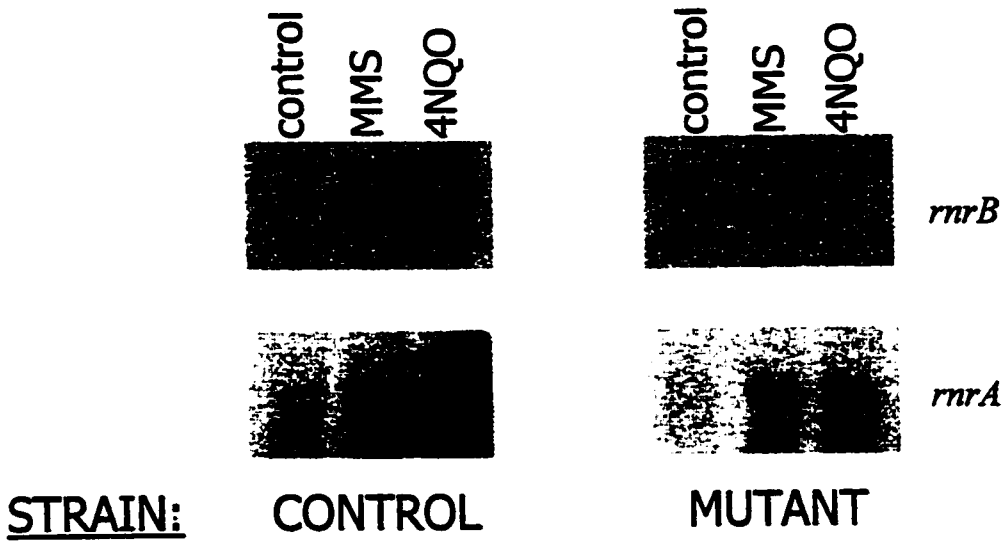


FIGURE 15. Effect of DNA-damaging agents on the accumulation of the *rnrB* transcript in RnrB-P-KO mutants.

RnrB-P-KO cells were treated with 10 mM MMS or 5 μg/ml 4NQO for 1 h. RNA was extracted, resolved by denaturing electrophoresis, and blotted onto membranes. Autoradiographs obtained from probing the same membrane for the *rnrB* gene and the *rnrA* gene are shown.

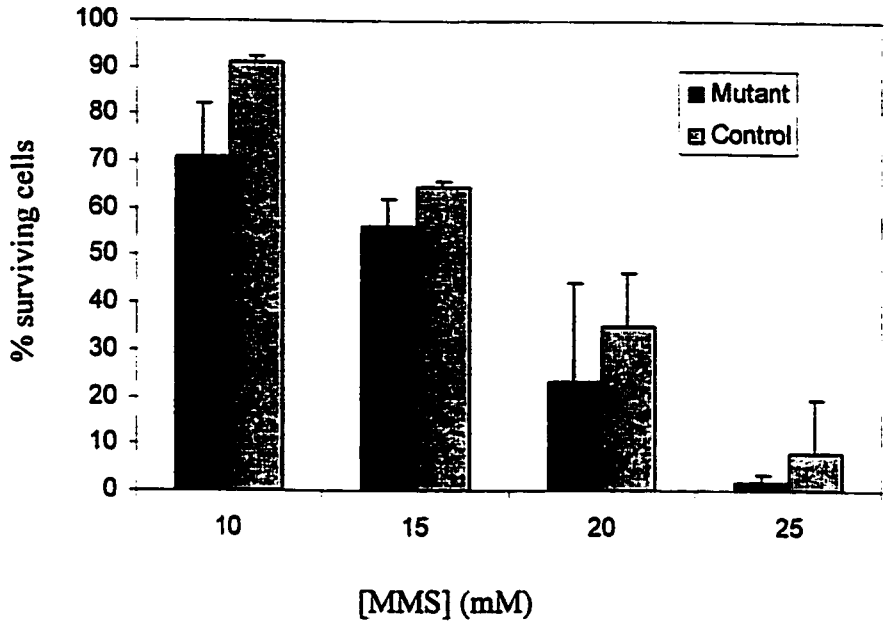


FIGURE 16. Survival of the RnrB-P-KO strain to DNA-damaging agents. Vegetatively growing cells were treated with various doses of MMS for 1 h. Aliquots of the cultures were spread on SM plates with *Enterobacter aerogenes* and incubated at room temperature until plaques appeared. The number of plaques formed was counted. The results are expressed in terms of proportion of plaques recovered in the treated cells compared to that of untreated controls. The errors represent the standard deviation of at least 2 independent experiments.

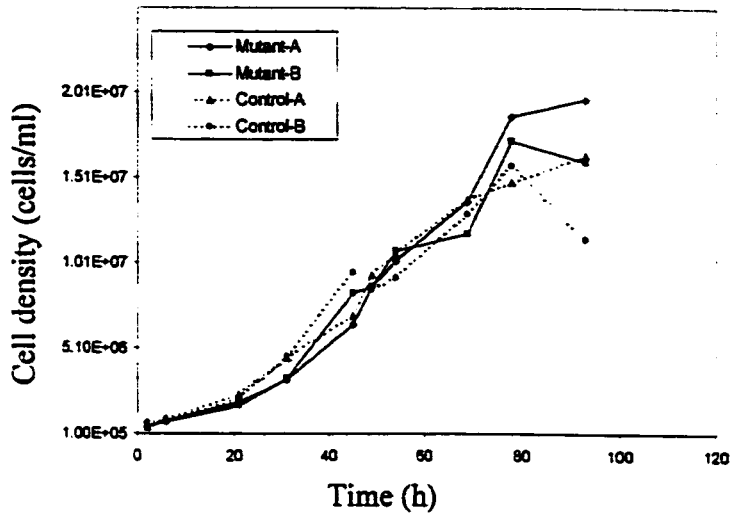
The next experiment done was to determine the rate of cell growth after treatment with DNA-damaging agents. Cells were treated with MMS for 1 h, washed and resuspended in growth medium again. The number of cells in control cultures as well as in cultures treated with 10 or 20 mM MMS was monitored directly using a hemacytometer for 4-5 days (Figure 17). In untreated cells as well as cells treated with 10 mM MMS, the growth rates between the control and the RnrB-P-KO strain were similar. At 20 mM MMS, however, a dramatic difference was observed: cell numbers for the control strain did not increase for the first 24 h, after which cell number started to increase. In contrast, the mutant strain showed no increase in cell number for the first 48 h after treatment. Moreover, the rate of increase in cell number appears to be appreciably slower than that of the control strain. Similar results were obtained in two other independent experiments (data not shown).

The number of live cells in these cultures was also determined by spreading on SM plates (Figure 18). The curve obtained for the number of live cells corresponded closely to that of the total number of cells, indicating that most cells in these cultures are alive.

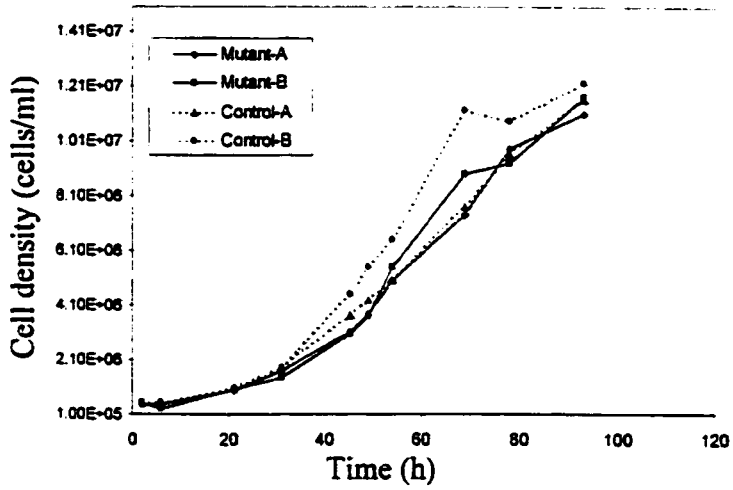
FIGURE 17. Growth rates of the rnrB-P-KO mutant strain and the control strain following treatment with MMS.

Determination of the growth rate of the RnrB-P-KO mutant strain versus that of the control strain in the absence of mutagens (untreated) or after a 1 h treatment with 10 or 20 mM MMS. Cell numbers were monitored by counting the cells with a hemacytometer. The experiment was performed in duplicate (A and B).

Untreated



10 mM MMS



20 mM MMS

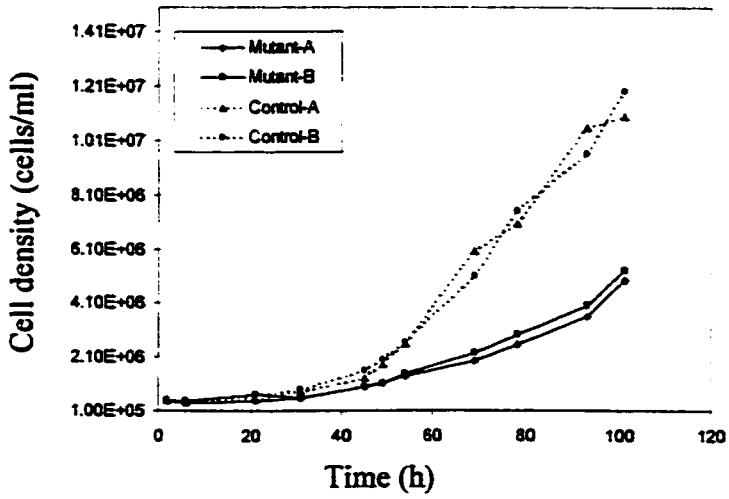
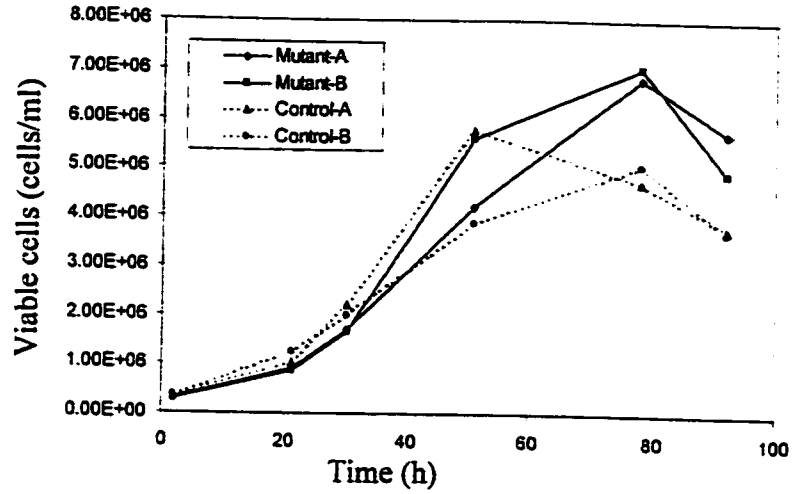


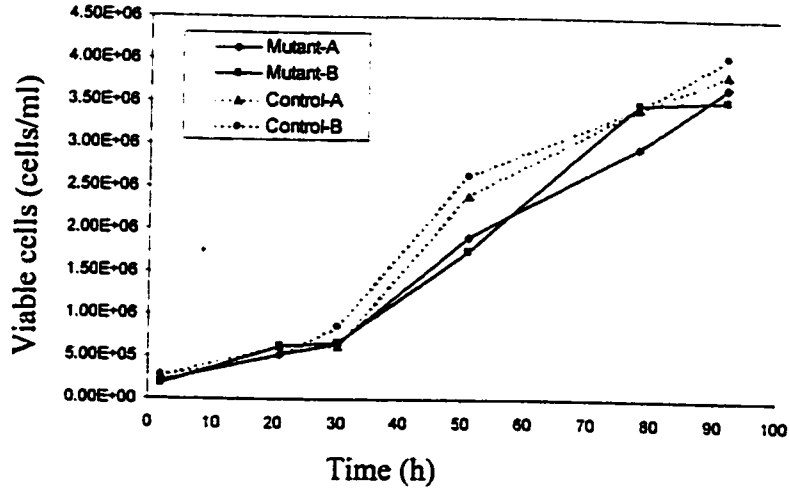
FIGURE 18. Number of viable cells in RnrB-P-KO and control cultures treated with MMS.

Aliquots from the cultures in Figure 17 were spread on SM plates with *Enterobacter aerogenes* and incubated at room temperature until plaques appeared. The number of plaques formed was counted. The number of viable cells in the culture is shown (cells/ml).

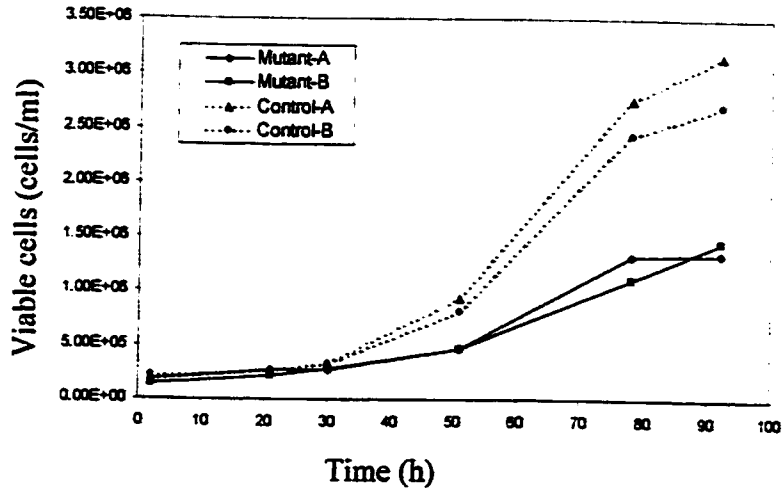
Untreated



10 mM MMS



20 mM MMS



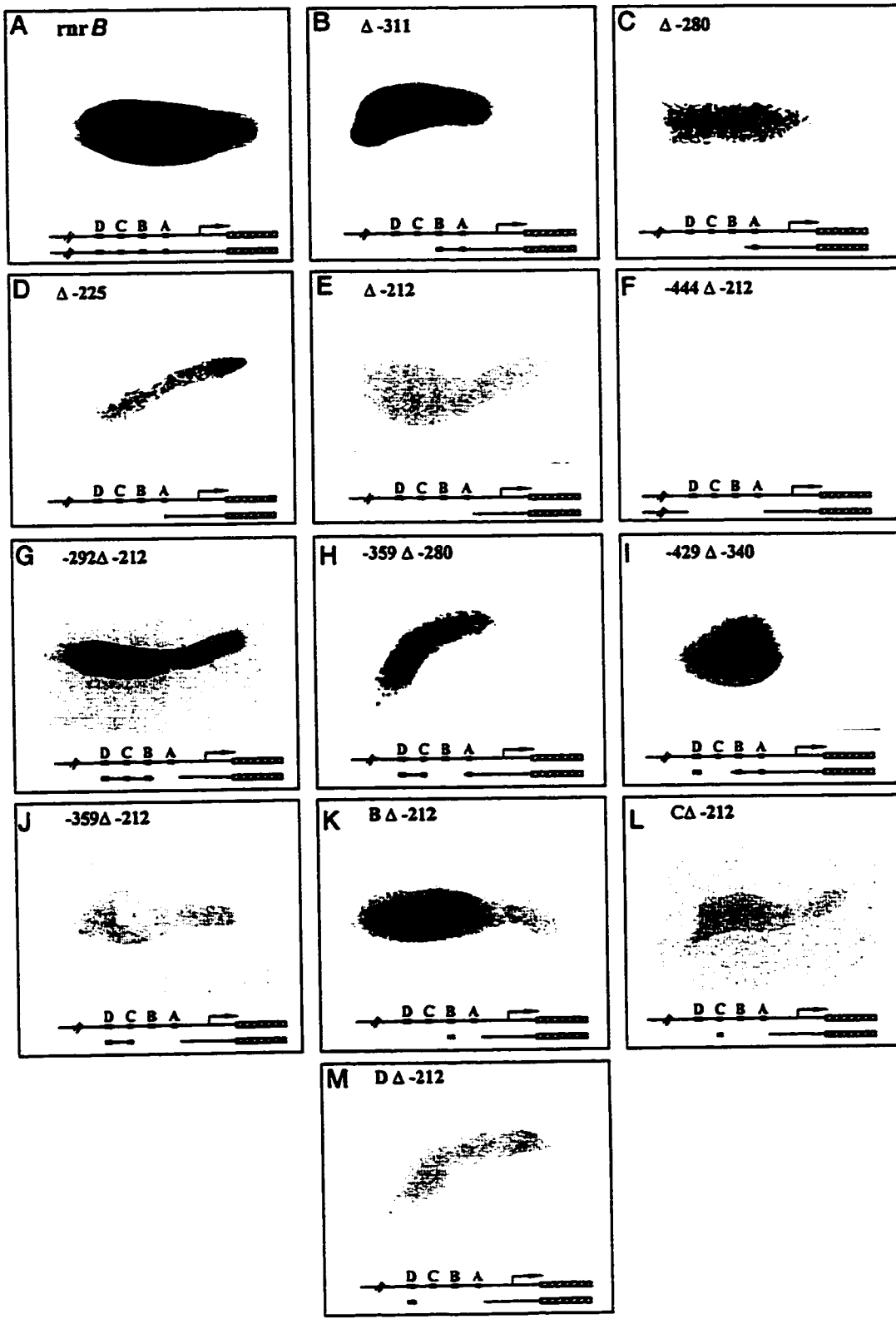
3.4. Developmentally-regulated expression of *rnrB*

3.4.1. Identification of *cis*-acting elements controlling the developmental expression of *rnrB*

Previously our laboratory has shown by histochemical staining that the *rnrB* gene is expressed only in the posterior, prespore zone (Tsang *et al.*, 1996; Figure 19A). To identify the *cis*-acting elements responsible for the cell-type-specific expression of *rnrB*, we used the 5' deletion constructs described in Section 3.3.8. Cells transformed with the 5' deletion construct Δ-450 had a staining pattern that was indistinguishable from that of the undeleted control (data not shown). Therefore, subsequent deletion analysis focused on the region downstream of -450. The internal deletion constructs used were similar to those described in Section 3.3.8 except that they do not contain any sequence upstream from the *Xba*I site (-450 relative to the ATG) (Bonfils *et al.*, 1999). Transformants bearing these different constructs were developed to the slug stage and stained for β-galactosidase activity. Figure 19 shows the results of these experiments. Deletion constructs containing both box A and box B (Δ-311 and -429Δ-340) displayed prespore-specific expression of β-galactosidase activity at the slug stage similar to that of the undeleted construct (Figure 19A, B, I). Constructs carrying either box A or box B alone (Δ-280, -292Δ-212, -359Δ-280 and BΔ-212) exhibited β-galactosidase activity primarily in the prespore zone with some activity in the prestalk region (Figure 19C, G, H, K). Interestingly, slugs developed from cells carrying Δ-225, containing the proximal half of box A, exhibited random distribution of β-galactosidase activity, with high levels of expression in the prestalk zone (Figure 19D).

FIGURE 19. Histochemical staining of β-galactosidase activity of *Dictyostelium* cells transformed with various RnrB-lacZ constructs.

Cells transformed with various RnrB-lacZ constructs were developed for 16-18 h to the migrating slug stage. The slugs were fixed and assayed histochemically for β-galactosidase activity. *Panel A* shows the staining pattern of a slug developed from cells which had been transformed with an undeleted construct. *Panels B-M* display the staining patterns of slugs whose cells harbour the deletion constructs indicated in the panels. In each panel, the anterior or prestalk region of the slug is pointing toward the right side. Also shown in the bottom part of each panel is the schematic presentation of the borders of the deletion.


A rnrB
D C B A
B Δ-311
D C B A
C Δ-280
D C B A
D Δ-225
D C B A
E Δ-212
D C B A
F -444 Δ-212
D C B A
G -292Δ-212
D C B A
H -359 Δ-280
D C B A
I -429 Δ-340
D C B A
J -359Δ-212
D C B A
K BΔ-212
D C B A
L CΔ-212
D C B A
M DΔ-212
D C B A

The internal deletion constructs that are missing both boxes A and B, Δ-212, -444Δ-212, -359Δ-212, CΔ-212, and DΔ-212, displayed very low level of β-galactosidase scattered throughout the slugs (Figure 19E, F, J, L and M) (Bonfils *et al.*, 1999). Taken together, these results indicate that box A and box B play a major role in the developmentally-regulated expression of *rnrB* in prespore cells.

3.4.2. Mutational analysis of the prespore-specific element box A

To refine the mapping of the elements required for *rnrB* expression we have performed mutational analysis on the box A element, which we have shown to be important for prespore-specific expression (Bonfils *et al.*, 1999; see Figure 19). Two mutations were introduced in the RnrB-ile-αpgal construct (Δ-450-ile-αpgal): A1 and A2, shown in Figure 20. Transformants bearing these constructs were developed to the slug stage and stained for β-galactosidase activity. The Δ-450/A2-ile-αpgal construct directed normal prespore staining. Interestingly, the –Δ450/A1-ile-αpgal mutant showed predominantly prespore staining, but with some expression in the prestalk zone, similar to constructs containing only box A or box B (Δ-280, -292Δ-212, -359Δ-280 and BΔ-212).

3.4.3. Effect of a genomic deletion of the cell-type-specific response elements

As described in Section 3.3.9, the RnrB-P-KO mutant harbours a deletion of all four G/C-rich boxes of the *rnrB* promoter, box A, box B, box C and box D. Since box A and box B are required for *rnrB* expression in developing cells, we predicted that the

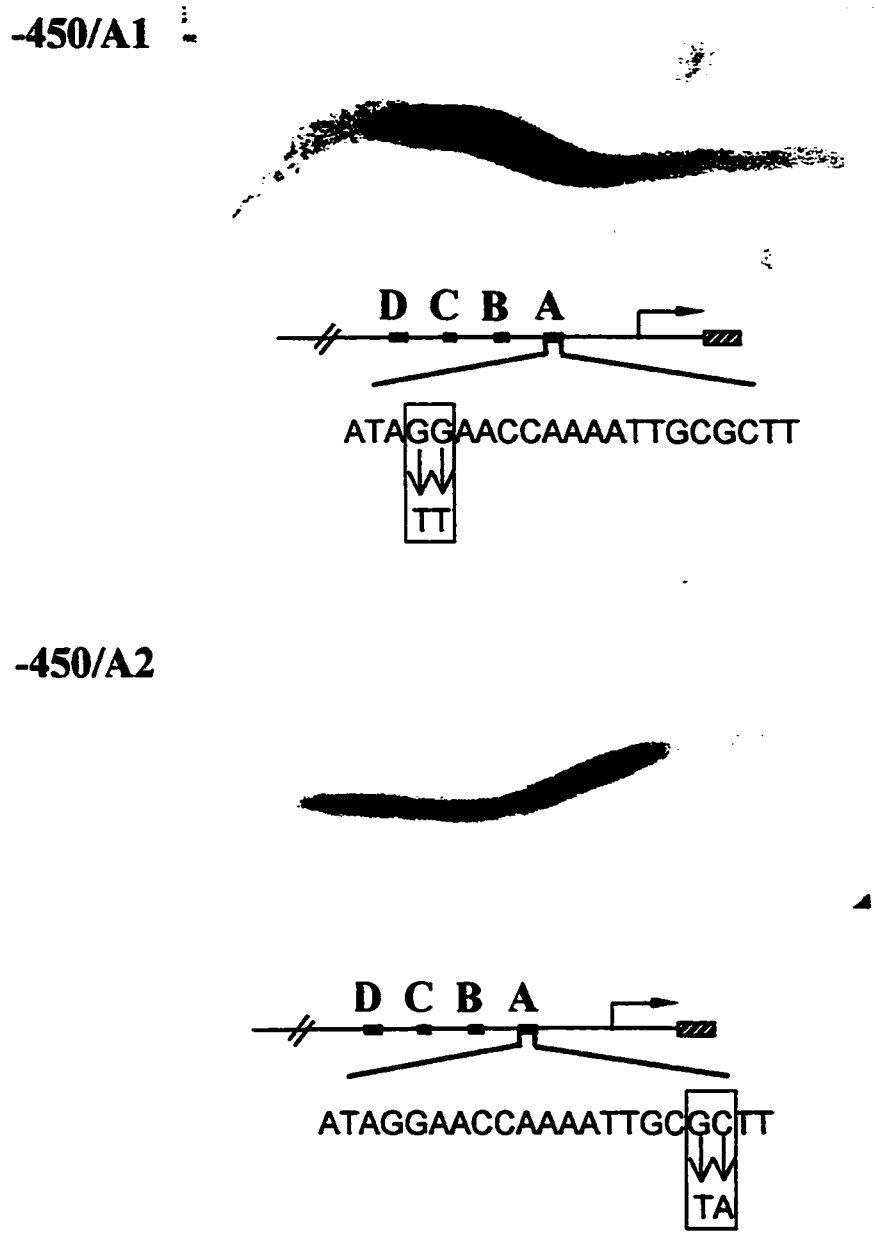


FIGURE 20. Histochemical staining of β-galactosidase activity of cells transformed with constructs bearing mutations in the box A element.

Cells transformed with Δ-450/A1 or Δ-450/A2 constructs were developed for 16-18 h to the migrating slug stage. The slugs were fixed and assayed for β-galactosidase activity histochemically. Also shown in the bottom part of each panel is a diagrammatic representation of the mutations.

mutant would also be unable to express *rnrB* in developing cells. To test this, we performed Northern analysis on RNA extracted from vegetative cells (0) and cells developed for 4, 8, 12 or 16 h. Figure 21 shows that the control strain (a clone resistant to blasticidin but in which the *rnrB* locus is intact) exhibited a profile of *rnrB* expression similar to that of the parental AX2 strain. The RnrB-P-KO strain failed to express *rnrB* in late developing cells, but exhibited expression in vegetative cells.

Many studies have shown that there is nuclear DNA synthesis in prespore cells of the slug stage, from 14-16 h of development (Zimmerman and Weijer, 1993; Deering, 1982; Durston and Vork, 1978; Zada-Hames and Ashworth, 1978). Presumably, the reason *rnrB* is expressed at this stage is to provide precursors required for DNA replication. If this round of DNA replication is necessary for completion of development, we expected that the mutant would show abnormalities in the developmental process. However, the data we have obtained so far indicate that the lack of expression of *rnrB* in late development has no effect on *Dictyostelium* development. We did not observe any morphological defects in the developing structures. Also, the developmental program of the mutant spanned 24 h, as for the untransformed and the control strains.

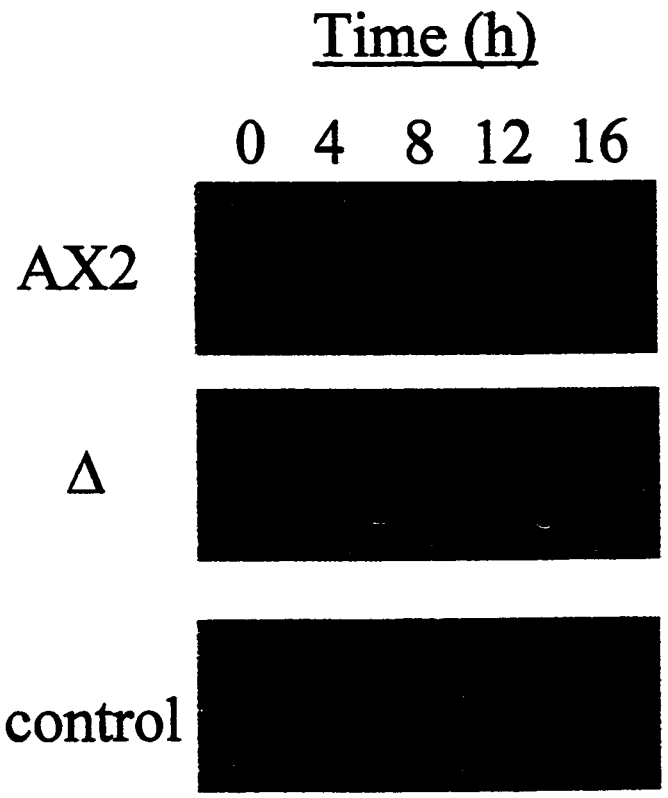
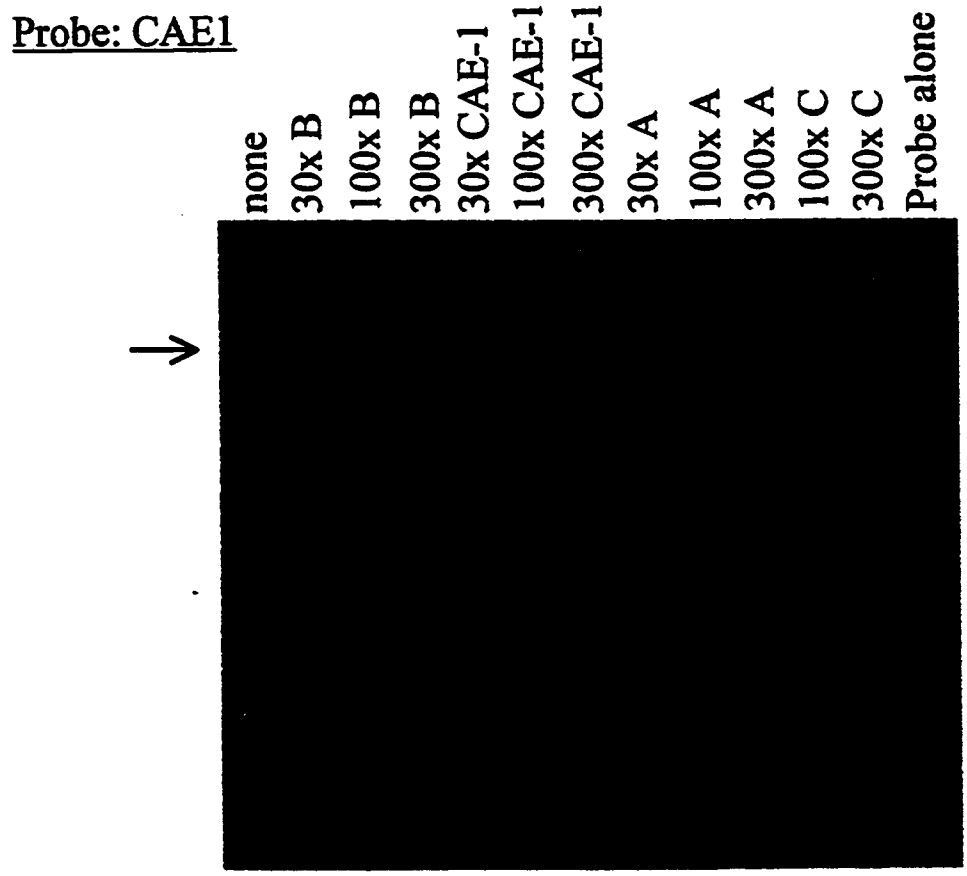


FIGURE 21. Expression of *rnrB* in AX2, RnrB-P-KO and a control strain. Total cellular RNA was extracted from vegetative cells as well as from cells developed for 4, 8, 12 or 16 h. The RNA was resolved on denaturing agarose gels and blotted onto nylon membranes. The autoradiographs were obtained from probing the membranes with the *rnrB* gene.

3.5. Factors regulating the expression of *rnrB*

3.5.1. GBF, a known transcription factor, does not bind the *rnrB* promoter

The mechanisms regulating the expression of prespore genes are largely unknown. Since the results obtained with the deletion analysis of the *rnrB* promoter indicate that box A and box B are responsible for the cell-type-specific expression of *rnrB*, we decided to identify the transcriptional factor(s) that bind to these elements. Comparing these sequences with transcription factor binding sites revealed that the sequence of box B harbours half of a CAE box (see Figure 22) found in the promoters of several cAMP-induced genes (Powell-Coffman and Firtel, 1994; Haberstroh and Firtel, 1990; Fosnaugh and Loomis, 1993; Esch and Firtel, 1991; Ceccarelli *et al.*, 1992; Hjorth *et al.*, 1990; Pavlovic *et al.*, 1989). The CAEs interact specifically with the *trans*-acting factor GBF (Hjorth *et al.*, 1989), with the element CAE-1 of *cotC* displaying the highest affinity (Hjorth *et al.*, 1990). We tested the ability of box B to compete for GBF using electrophoretic mobility shift assay according to Schnitzler *et al.* (1994). Using oligonucleotides corresponding to CAE-1, we detected a high molecular weight factor that appears to correspond to the GBF protein (Figure 22). A 30-fold excess of unlabelled CAE-1 was sufficient to completely compete for binding of labelled CAE-1 to GBF (Figure 22, lane 5). Box A, box B, and box C were poor competitors. This indicates that GBF is unlikely to control the expression of *rnrB*.




```
CAE-1: 5'-TTTTTCACACA··CCCACACACTAATTTACCCCATTTTTG-3'
                      ||||||  |||
Box B: 3'-TAATACACACACATTCCCGAAAG-5'
```

FIGURE 22. Electrophoretic mobility shift assay testing the ability of the *rnrB* promoter elements to bind GBF.

Cytosolic extracts were prepared from cells developed for 15 h. An oligonucleotide corresponding to CAE-1 was used as a probe. The binding was competed with excess of unlabelled box B, CAE-1, box A or box C as indicated. The shift presumably produced by GBF is indicated by an arrow. The homology between CAE-1 and box B is also shown.

3.5.2. Cellular factors binding to box A

We used electrophoretic mobility shift assays using a radiolabelled oligonucleotide corresponding to box A to identify factors that bind to the *rnrB* promoter elements conferring prespore-specific expression. We detected a specific complex with nuclear extracts prepared from cells developed for 15 h (Figure 23). A 300-fold molar excess of oligonucleotides corresponding to either box B, box C, box D or CAE-1 did not compete for binding of box A in the fast-migrating complex, indicated by the arrow (Figure 23) (Bonfils *et al.*, 1999).

We expected that the transcription factors regulating the developmental expression of *rnrB* should themselves have developmentally-regulated activity. Nuclear and cytosolic extracts were prepared from vegetatively growing cells, and from cells developed for 6 h, 12 h, 15 h and 18 h. Oligonucleotides corresponding to box A bind a nuclear factor that was undetectable in nuclear extracts prepared from vegetative cells or from cells developed for 6 h. This factor was present in cells developed for 12 h, 15 h and 18 h (Figure 24). The presence of this activity in cytosolic extracts could not be assessed because of the presence of a nonspecific complex with similar mobility on the gel-shift assay (data not shown).

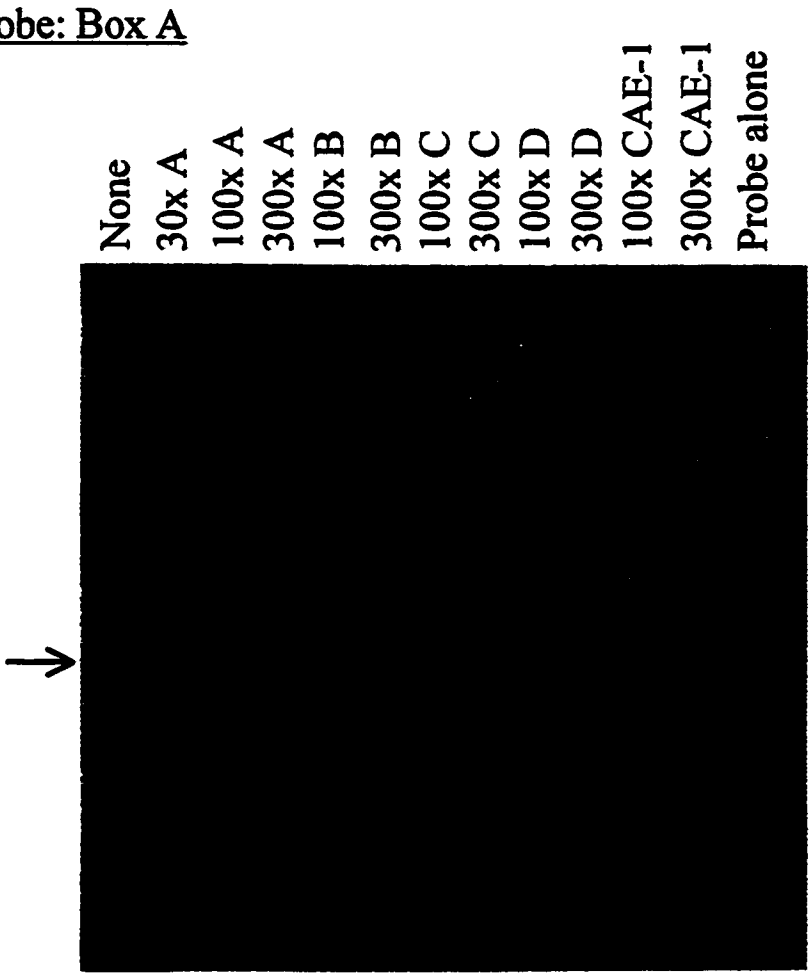


FIGURE 23. Electrophoretic mobility shift assay with box A in the presence of unlabelled competitors.

Nuclear extracts prepared from cells that had been developed for 15 h were mixed with labelled oligonucleotide corresponding to box A (far left lane) or mixed with labelled box A in the presence of excess unlabelled oligonucleotides corresponding to box A, box B, box C, box D or CAE-1 as indicated. The specific factor is marked by an arrow.

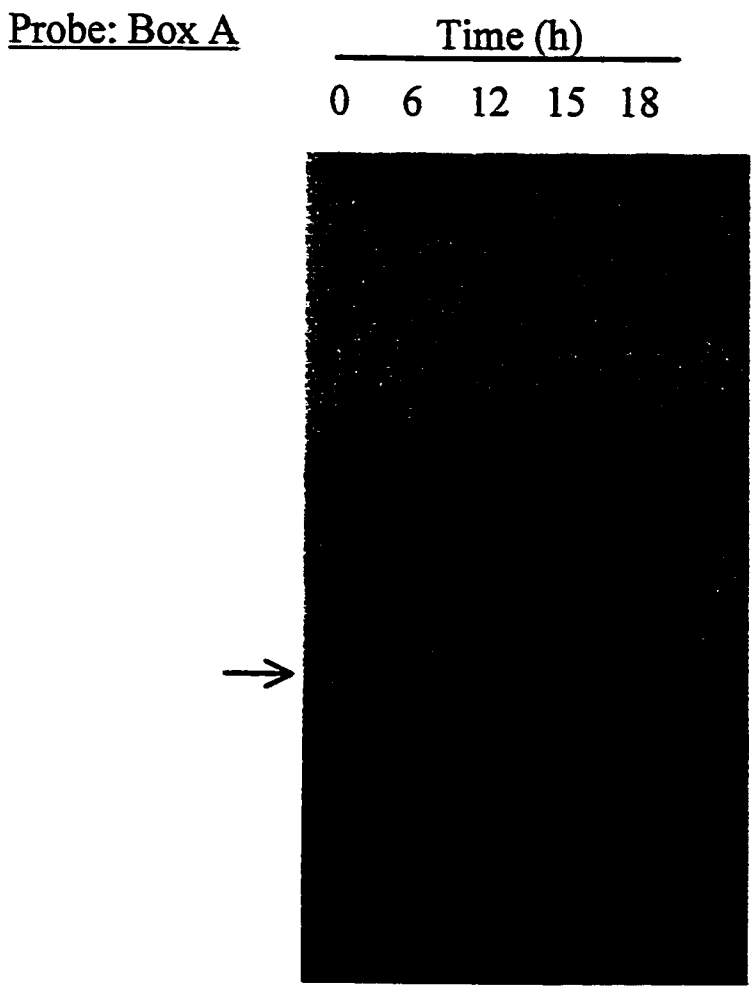


FIGURE 24. Electrophoretic mobility shift assay showing the developmental regulation of the box A binding factor.

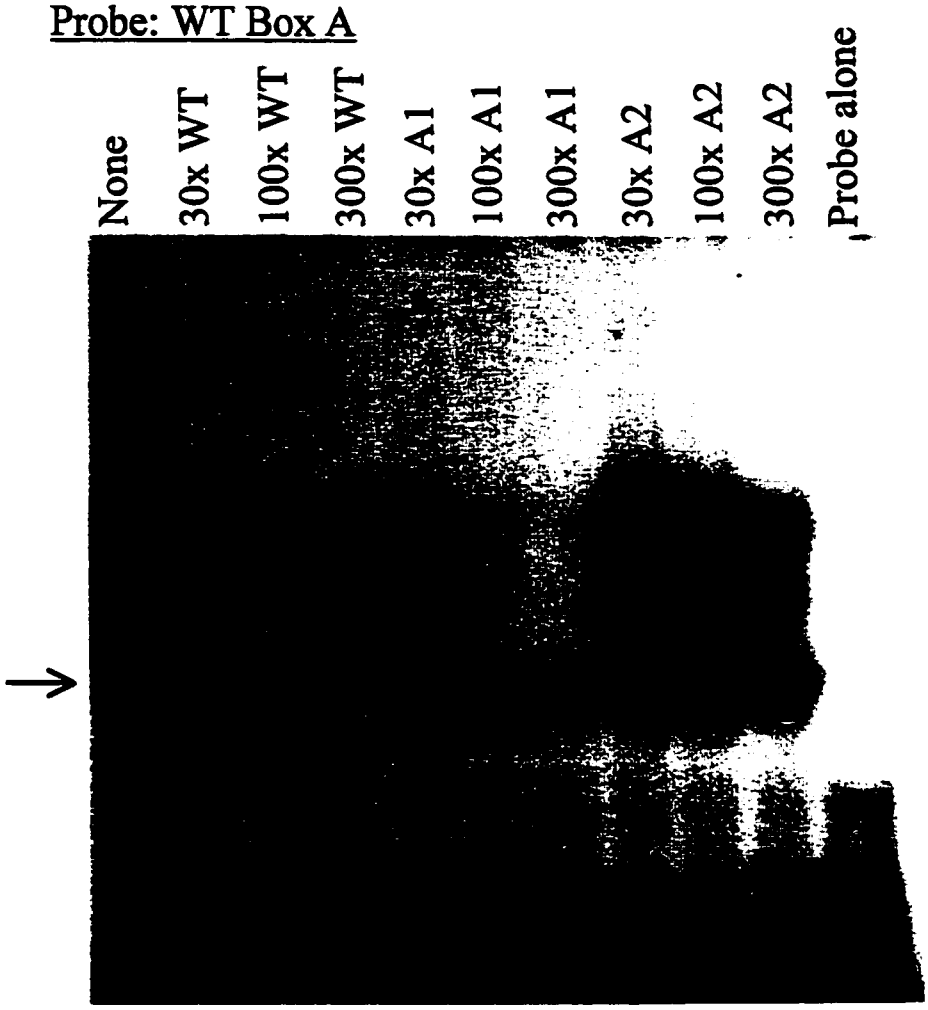
Nuclear extracts were prepared for vegetative cells, 0 h, or from cells that had been developed for 6, 12, 15 or 18 h. The nuclear extracts were mixed with labelled oligonucleotide corresponding to box A and resolved on non-denaturing polyacrylamide gel. The arrow marks the position of the specific complex.

We also tested the ability of two oligonucleotides containing mutations in box A, A1 and A2, to compete with the developmentally regulated nuclear factor that binds box A (see Figure 24). Figure 25 shows the results of this experiment. The A1 mutant was able to compete with the box A binding factor in a manner similar to wild-type box A. However, the A2 oligonucleotide had a significantly reduced affinity for the box A binding factor. These results suggest that the GCGC sequence near the 3' end of box A is important for recognition by the box A binding factor detected by this assay.

3.5.3. Cellular factors binding to box B

Figure 26 shows the presence of a cytosolic factor that exhibits binding to the box B oligonucleotide. This factor was effectively competed with unlabelled box B, but poorly with unlabelled boxes, A, C or D. Competition experiments showed that a 30-fold box B was about as effective a competitor as 300-fold excess of CAE-1 (compare lanes 2 and 7 in Figure 26). These results suggest that although box B has homology to the CAE elements, it binds to different factors.

We investigated the developmental regulation of the box B binding factor. It was present in cytosolic extracts from vegetatively growing cells as well as cells from all stages of development (Figure 27). A complex of box B with the same electrophoretic mobility was detected in nuclear extracts. This factor was not detected in the nuclear fractions of vegetative cells or early developing cells, but was present in the nuclear extracts of cells that had been developed for 12 h or more (Figure 28).



WT box A: 5'-ATAGGAACCAAAATTGCGCTT-'3

box A1: 5'-ATATTAACCAAAATTGCGCTT-'3

box A2: 5'-ATAGGAACCAAAATTGCTATT-'3

FIGURE 25. Electrophoretic mobility shift assays with box A in the presence of unlabelled A1 and A2.

Nuclear extracts prepared from cells that had been developed for 15 h were mixed with labelled oligonucleotide corresponding to box A (far left lane) or mixed with excess unlabelled oligonucleotides corresponding to box A, or the A1 and A2 versions of box A as indicated. The developmentally-regulated factor is marked by an arrow.

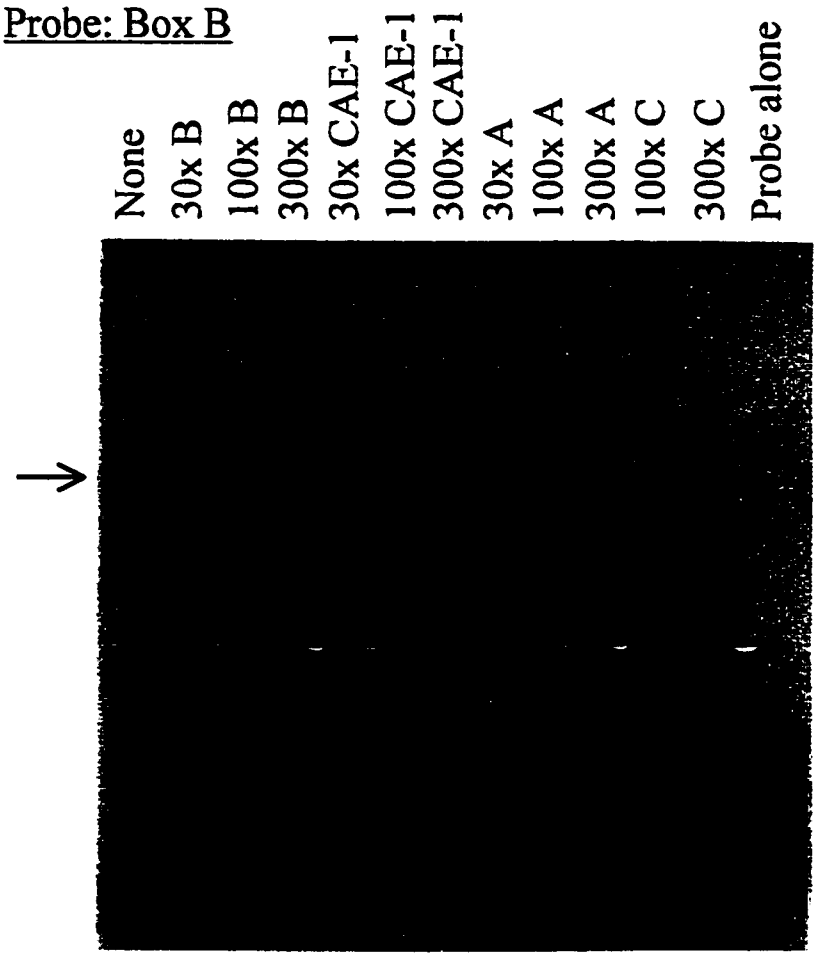


FIGURE 26. Electrophoretic mobility shift assay with box B in the presence of unlabelled competitors.

Cytosolic extracts were prepared from cells developed for 15 h. An oligonucleotide corresponding to box B was used as a probe. The binding was competed with excess of unlabelled box B, CAE-1, box A or box C as indicated. The specific complex is indicated by an arrow.

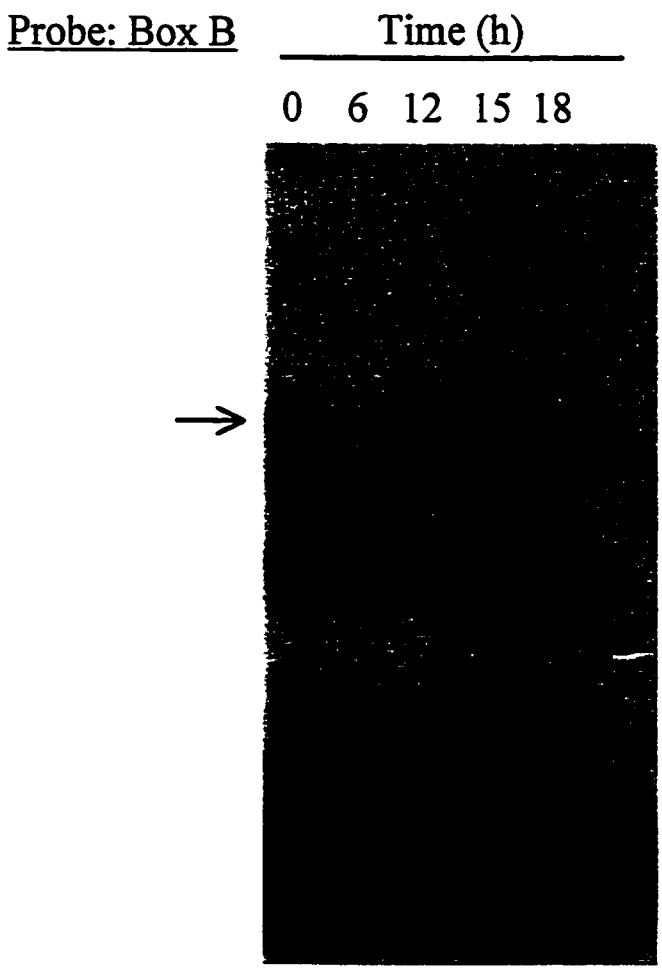


FIGURE 27. Electrophoretic mobility shift assay showing the activity of the box B binding factor during the *Dictyostelium* life cycle.

Cytosolic extracts were prepared for vegetative cells, 0 h, or from cells that had been developed for 6, 12, 15 or 18 h. The extracts were mixed with labelled oligonucleotide corresponding to box B and resolved on non-denaturing polyacrylamide gel. The arrow marks the position of the specific complex.

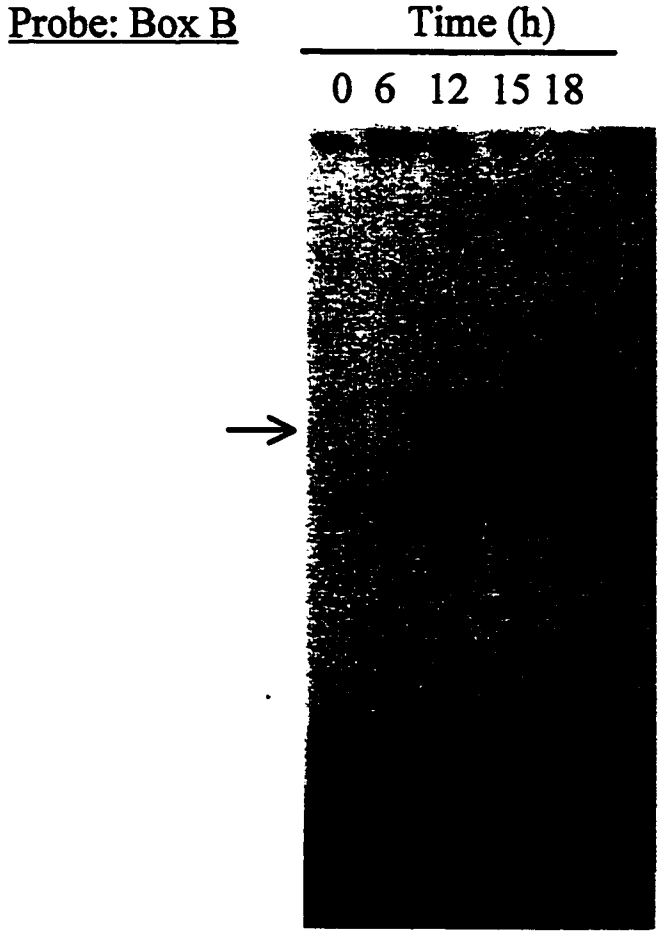


FIGURE 28. Electrophoretic mobility shift assay showing the developmental regulation of the box B binding factor in nuclear extracts.

Nuclear extracts were prepared for vegetative cells, 0 h, or from cells that had been developed for 6, 12, 15 or 18 h. The nuclear extracts were mixed with labelled oligonucleotide corresponding to box B and resolved on non-denaturing polyacrylamide gel. The arrow marks the position of the specific complex.

4. DISCUSSION

4.1. Cell cycle regulation of *rnrB*

We have shown that the *rnrB* gene is expressed in a regulated fashion during the cell cycle in *Dictyostelium*. The results presented here indicate that cold synchronized cells express *rnrB* in late G2, while cells synchronized by the high density method express *rnrB* in mid-G2. Since the two types of cell cycle synchronization experiments gave different results, we examined the activity of the *rnrB* promoter in unsynchronized cells. In results presented elsewhere, we have measured the proportion of BrdU-positive cells that also exhibited β-galactosidase activity (MacWilliams *et al.*, 2001). Cells were either exposed continuously to BrdU or for 30 minutes before the beginning of the experiment. Both continuous- and pulse-labelling experiments suggest that *rnrB* is expressed during two periods of the cell cycle in asynchronous cells. The results obtained with these experiments indicate that few cells express the *lacZ* reporter gene between 2 and 5 h after S phase, or in the last 3 h of the cycle. One period is close to S phase and may well correspond to the expression maximum seen in cold-synchronized cells. The other peak, 6 h after S, corresponds roughly with the expression maximum observed in the high-density synchronization experiments.

The *rnrB* expression pattern in *Dictyostelium* therefore departs dramatically from that in other organisms studied to date. In general, the genes encoding either the small or the large subunit of RNR, sometimes both, show cell cycle regulation, and the expression inevitably occurs either at the G1/S boundary or in early S (reviewed in 1993 by Elledge *et al.*). The late G2 expression in *Dictyostelium*, which occurs shortly before S-phase, corresponds functionally to the G1/S expression in other systems. The role of the mid-G2

expression of *rnrB* is presently unknown. We are presently testing whether this profile of expression is specific to *rnrB*. Our preliminary results indicate that *rnrA*, encoding the large subunit of ribonucleotide reductase, is regulated in a fashion similar to that of *rnrB* following synchronization (H. MacWilliams and P. Gaudet, unpublished observations) and may therefore apply to several other genes.

In yeast and in mammalian cells G1 cyclins activated by their respective cyclin-dependent kinases activate transcription factors involved in cell-cycle-regulated gene expression, SBF and MBF in yeast, and E2F in mammalian cells. These factors are inactive in late G2. It will be interesting to know whether similar pathways are used in *Dictyostelium*, or if the factors required for expression of S-phase genes are activated by homologs of G2 cyclins.

4.2. Response of *rnrB* to DNA-damaging agents

4.2.1. Change in *rnrB* transcript level in response to DNA-damaging agents

In mammalian cells and in yeast, the level of expression of a large number of genes increases in response to DNA-damaging agents (Friedberg *et al.*, 1995; Jelinski and Samson, 1999; Jelinsky *et al.*, 2000). A number of these responsive genes are normally required for repair and synthesis of DNA. In *Dictyostelium*, the genes encoding apurinic/apyrimidic endonuclease and the helicases *repB* and *repD* (*repB* and *repD* are homologs of the xeroderma pigmentosum *XPB* and *XPD* genes, respectively) have been shown to be up-regulated by DNA-damaging agents (Freeland *et al.*, 1996; Yu *et al.*, 1998). We showed here that the transcript level of the small subunit of ribonucleotide reductase increased when *Dictyostelium* cells were treated with 4NQO, MMS, or UV

irradiation. These results suggest that, as in other organisms, DNA-damaging agents induce the expression of specific *Dictyostelium* genes involved in DNA repair and DNA metabolism (Gaudet and Tsang, 1999).

4.2.2. Response of *rnrB* to the inhibitor hydroxyurea

Hydroxyurea is a specific inhibitor of ribonucleotide reductase. It inactivates the tyrosyl free radical of the small subunit that is required for enzyme activity. Overexpression of the small subunit is sufficient to confer resistance to hydroxyurea in mammalian cell lines (Zhou *et al.*, 1995). The presence of hydroxyurea leads to the depletion of nucleotides, and consequently blocks DNA synthesis. The inhibition of DNA synthesis by hydroxyurea can be reversed by addition of nucleotides (reviewed in Reichard, 1988). Hydroxyurea causes an increase in *rnr2* transcript level in yeast (Huang and Elledge, 1997; Elledge and Davis, 1989, 1990; Hurd and Roberts, 1989) and in *E. coli* (Sitjes *et al.*, 1992). In contrast, in response to hydroxyurea, mammalian cells increase the rate of synthesis and the stability of both subunits of ribonucleotide reductase (McClarty *et al.*, 1988) but do not alter the levels of their transcripts (Filatov *et al.*, 1996). Our results indicate that in *Dictyostelium* hydroxyurea causes up-regulation of the *rnrB* transcript only in actively growing cells. Early developing cells, which are not undergoing DNA replication, do not respond to this drug. This suggests that hydroxyurea does not directly induce transcription of *rnrB*; rather, limiting dNTPs for replication may be the actual signal.

4.2.3. Regulation of the DNA-damage response

The mechanisms mediating the effects of DNA-damaging agents share common features. In yeast, the up-regulation of ribonucleotide reductase by DNA-damaging agents is mediated by pre-existing factors that do not require protein synthesis to become activated (Elledge and Davis, 1989). The expression of the apurinic/apyrimidic endonuclease gene from *Dictyostelium* has also been shown to be DNA damage-inducible in a protein-synthesis independent fashion (Freeland *et al.*, 1996). Our results with the protein synthesis inhibitor cycloheximide strongly suggest that the up-regulation of the *rnrB* transcript upon treatment with DNA-damaging agents takes place via a similar mechanism. Furthermore, the demonstration by deletion analysis that specific promoter regions are necessary for the induction by DNA-damaging agents suggests that an increase in transcription of the *rnrB* gene is an important part of the response.

In *Dictyostelium*, the up-regulation of gene activity in response to DNA-damaging agents is transient. Besides *rnrB*, this has been shown to be the case for the apurinic/apyrimidic endonuclease gene as well as the *repB* and *repD* genes (Freeland *et al.*, 1996; Yu *et al.*, 1998). The drop in transcript level after prolonged treatment observed for these genes is possibly caused by cell death and/or breakdown of the drugs. In budding yeast the induction of a number of DNA damage-responsive genes has also been shown to be transient, including *RNR2, POL1, RAD6, RAD7, RAD18, RAD23* and *RAD51* (Elledge and Davis, 1989; Johnston *et al.*, 1987; Madura *et al.*, 1990; Jones *et al.*, 1990; Jones and Prakash, 1991; Madura and Prakash, 1990; Shinohara *et al.*, 1992).

The level of *rnrB* expression fluctuates during the life cycle of *Dictyostelium* (Tsang *et al.*, 1996). Regardless of the endogenous *rnrB* level, similar magnitude of

induction by DNA-damaging agents was observed during growth and at different stages of development. This suggests that the factors involved in the response to DNA-damaging agents are present at all stages of the life cycle. It also implies that the mechanisms mediating the effects of DNA-damaging agents on *rnrB* operate independently from processes that regulate the expression of *rnrB* during growth and development. In support of this finding, the *repB* and *repD* genes have also been shown to be induced by DNA-damaging agents at different stages of the *Dictyostelium* life cycle (Yu *et al.*, 1998; Gaudet and Tsang, 1999).

4.2.4. The *rnrB* promoter as an inducible expression system for *Dictyostelium*

The high, rapid up-regulation of the *rnrB* gene in response to DNA-damaging agents suggested that it could potentially be used as a controllable gene expression system. Inducible promoters are widely used for the analysis of gene function. Ideally, an inducible promoter vector should have the following characteristics: (i) low basal expression level, (ii) high expression following induction, (iii) rapid inducibility, and (iv) inducibility under all physiological conditions. Unfortunately, few inducible promoter systems are available in *Dictyostelium*, and although they provide high up-regulation of the gene of interest, the induction is slow and these systems can only be used with vegetative cells (Blaauw *et al.*, 2000; Blusch *et al.*, 1992).

We have shown that a DNA fragment comprising 450 bp of the *rnrB* promoter can confer DNA-damage responsiveness on two heterelogous genes, GFP and *lacZ* (Figures 10, 11 and 12; Gaudet *et al.*, 2001). Moreover, in collaboration with Dr. Gerald Weeks from the University of British Columbia we have demonstrated that

overexpression of the rasG protein under the control of the *rnrB* promoter resulted in increased rasG protein and activated potential rasG downstream signalling pathways, a physiological response similar to that observed with another controllable gene expression system, the discoidin system (Secko *et al.*, in press).

The fact that the drugs used for the control of gene expression with the pRNR-P system are DNA-damaging agents raises concern about the possible induction of mutations. However, the number of mutations caused by the doses required to induce expression from the *rnrB* promoter is relatively low. Over 95 % of AX2 cells survive an exposure to 30 J/m^2 UV light (Gaudet and Tsang, 1999), which has been reported to result in a less than 2-fold increase in the number of mutations compared to the spontaneous mutation level (Pogdorski and Deering, 1980). For a 1 h treatment with 10 mM MMS, the survival rate is about 70 % (Gaudet and Tsang, 1999), which corresponds to approximately a 3.5-fold higher mutation frequency than the spontaneous mutation level (Pogdorski and Deering, 1980) (Gaudet *et al.*, 2001).

We have investigated the effects of DNA-damaging agents on development and found that the doses required for induction caused little or no detectable effects on the progress of development and on the number and size of fruiting bodies. No adverse effects were observed when cells were treated with 30 J/m^2 UV. Less than a 1-h delay in development was observed when cells were treated with 100 J/m^2. At 10 μg/ml or lower concentrations, 4NQO did not alter the profile of development even when the treatment was sustained throughout the 24-h developmental cycle. Treatment with 10 mM MMS during the first 2 h of development caused a significant delay in development. However, no defects were observed when the cells were treated after this critical period for a

duration of less than 2 h (Gaudet *et al.*, 2001). The fact that prolonged exposures to 4NQO shows no effect on development may be due to detoxification of the drug.

4.2.5. Promoter elements driving the DNA damage response in *rnrB*

The demonstration that a 450-bp promoter fragment was sufficient to drive GFP and *lacZ* expression shows that this sequence contains all the information necessary to confer DNA-damage-induced expression. This does not rule out the possibility that the introns play a role in the regulation as in the case of mammalian cells (Tanaka *et al.*, 2000); however, the introns are not essential for the response.

We have mapped the promoter elements required for response to MMS and 4NQO. To our knowledge, this is the first report that shows the involvement of different *cis*-regulatory elements in the response to different DNA-damaging agents. We observed that the *cis*-regulatory element box C was able to confer transcriptional response to both MMS and 4NQO. We also showed that box A mediated the response to MMS whereas box D promoted the response to 4NQO. The reason for the involvement of different *cis*-regulatory elements is not clear. These two drugs have different modes of actions, MMS induces alkylation of DNA while 4NQO generates bulky adducts (Friedberg *et al.*, 1995). The damage caused by these two drugs could activate different, yet overlapping, sets of transcription factors. Another possibility is that the damaged DNA is not the only cause of the response. Methyl methane sulfonate can generate alkylation damage to cellular components other than DNA, and 4NQO is known to cause oxidative stress. These other types of damage could trigger signal transduction cascades that result in the expression of the *rnrB* gene in addition to the DNA damage signal.

The results obtained from promoter analysis are complex, especially those involving internal deletions. There are several possible reasons for some of the inconsistencies observed. Spacing between regulatory elements may influence the level of expression. For example, the internal deletion construct containing both box A and box B (-444Δ-311) was induced to a higher level than the construct containing only box A (-444Δ-280). But in the 5' deletions, the construct containing both box A and box B (Δ-311) exhibited a similar level of induction as the construct containing box A alone (Δ-280) (Gaudet and Tsang, 1999).

4.2.6. Physiological role of the DNA damage induction

Repair of damaged DNA requires that dNTPs be produced outside of the S phase of the cell cycle. Work from other groups indirectly shows that dNTPs are limiting for the DNA damage response in *S. cerevisiae*. Deletion of the *sml1* gene, encoding a negative regulator of ribonucleotide reductase activity (Chabes *et al.*, 1999) increases levels of dNTPs and survival to hydroxyurea and MMS (Zhao *et al.*, 1998). Mutations in the genes encoding the Rad53p and Mec1p kinases, involved in the signalling leading to transcriptional activation responses following DNA damage, can be rescued by overexpressing the ribonucleotide reductase large subunit (Desany *et al.*, 1998). Tanaka and collegues (2000) have recently used antisense RNA to prevent the up-regulation of one of the genes encoding the small subunit of ribonucleotide reductase following DNA damage in mammalian cells. They have observed decreased rates of incorporation of radiolabelled nucleotides into genomic DNA (presumably indicative of repair synthesis), prolonged cell cycle arrest and decreased cell viability following irradiation. They

concluded that ribonucleotide reductase is required to feed DNA synthesis for DNA repair.

The results we have obtained with a mutant having a genomic deletion of the DNA-damage response elements of the *rnrB* promoter are consistent with these studies. Our results showed that the absence of induction of the ribonucleotide reductase small subunit interferes with the recovery from DNA damage stress. After treatment with 20 mM MMS, we observed a longer lag before cell number increase resumed in the RnrB-P-KO strain compared with the control strain (Figures 17 and 18). In the control strain, when cells began to divide they proceeded at a rate resembling that of untreated cultures. In contrast, the increase in cell numbers for the mutant was very slow. In these experiments we may be measuring two different effects: cell cycle arrest and cell death. However, since most cells counted were alive (compare Figures 17 and 18), we can infer that the reduced rate of growth is mostly caused by cell cycle arrest.

These results may have important implications for understanding the mode of action of chemotherapeutic drugs. Most chemotherapeutic agents act by causing damage to DNA, which in turn presumably trigger cell death by apoptosis. Ribonucleotide reductase inhibitors are also used as anti-cancer drugs, often in combination with radiation or other chemotherapeutic agents (Tancini, 1998; Haraf *et al.*, 1997; Kinsella, 1992). The presumed mode of action of ribonucleotide reductase inhibitors and other inhibitors of DNA synthesis (Szekeres *et al.*, 1997; Yarbo, 1992) is that they interfere with cell division and therefore slow down tumor growth. Based on our results and those of Tanaka *et al.* (2000), it seems plausible that the most likely mode of action of

ribonucleotide reductase inhibitors in tumor cells is to reduce the efficiency of DNA repair and therefore indirectly promote apoptosis.

4.3. Developmental regulation of *rnrB*

4.3.1. Elements conferring cell-type-specific expression

How the expression of prespore-specific genes is regulated in *Dictyostelium* is not well understood. Consensus CAEs are found in promoters of most prespore genes studied to date (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al.*, 1994, Early and Williams, 1989). In most instances, the consensus CAEs have been shown to influence the level of gene activity but do not confer cell-type specificity. In general, removal of the CAEs from the promoter of prespore genes drastically reduces the level of expression but does not alter the spatial distribution of the residual activity (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al.*, 1994, Haberstroh *et al.*, 1991, Early and Williams, 1989). Also consistent with this idea is the observed function of GBF, the transcriptional activator that interacts with CAEs. Binding sites of GBF are found in the promoters of both prespore and prestalk genes (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al.*, 1994, Haberstroh *et al.*, 1991; Ceccarelli *et al.* 1992; Early and Williams, 1989; Pears and Williams, 1987; Datta and Firtel, 1987). Cells carrying a null mutation in GBF are arrested at the loose aggregate stage before cell-type-specific gene expression takes place (Schnitzler *et al.*, 1994). However, in certain contexts the CAEs have been shown to affect spatial expression. For example, mutations in one of three CAEs in the promoter of the prespore *cotC* gene can result in asymmetrical expression that is confined to the prespore zone (Haberstroh *et al.*, 1991, Haberstroh and Firtel,

1990). Another case is that of the *car3* gene. It is expressed throughout the slug and its promoter harbours two CAEs. Deletion of either of the two CAEs restricts expression to the prespore zone (Gollop and Kimmel, 1997). Taken together, these results suggest that interactions between GBF and transcriptional factors that bind *cis*-regulatory elements other than CAEs are involved in regulating the expression of cell-type-specific genes. The other *cis*-regulatory elements that may be involved include the A/T-rich elements identified in promoters of several genes expressed in post-aggregative cells (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al.*, 1994; Ceccarelli *et al.*, 1992; Esch *et al.*, 1992; Early and Williams, 1989; Datta and Firtel, 1988).

Results presented here suggest that the expression of *rnrB* in prespore cells involves mechanisms that are distinct from those regulating prespore-specific genes described previously. The three G/C-rich regions implicated in the expression of *rnrB* in postaggregative cells, boxes A, B, and D, bear no sequence similarity among themselves. Box D supports very low level of activity which is randomly distributed in the slug (Figure 19M). Boxes A and B can independently influence expression in prespore cells during postaggregative development. However, constructs containing only one of the two boxes exhibit lower activity than those carrying both boxes. In addition, these constructs show ectopic expression with activity transgressing to the prestalk zone (Figure 19C, G, H, K). Internal deletions missing both box A and box B display a very low level of activity in postaggregative cells, and the residual activity is randomly distributed in the slug (Figure 19J, M). Moreover, deletion of part of box A results in expression throughout the entire slug (Fig. 19D). These results suggest that prespore-specific expression of *rnrB* depends on the presence of both box A and box B (Figure 19A, B, I).

Unlike the CAEs, therefore, intact box A and box B play a direct role in specifying prespore expression. (Bonfils *et al.*, 1999).

Site-directed mutagenesis of box A of the *rnrB* promoter showed that the GG (or CC) dinucleotide near the 5' end of box A helps direct prespore-specific expression (Figure 20). This site could be similar to a site in the *cudA* gene that has also been shown to repress gene expression in prestalk cells. Site-directed mutagenesis of the CC dinucleotide sites shown below confers prestalk expression to constructs that showed prespore expression in the presence of the wild-type sequence (results presented here; Fukuzawa and Williams, 2000). Moreover, box B of the *rnrB* promoter may comprise a similar element. These results imply that the prespore-specific expression of *rnrB* is the result of repression in prestalk cells.

```
                    rnrB Box A:  ATAGGAACCAAAATT-------GCGC
                                   ||                    |||
cudA rep (reverse complement):  TTGGAAAAAAAATTTT---  CCCGC
                                  ||                   ||||
 rnrB Box B(reverse complement): GGAAAATACACACACATTCCCG
```

4.3.2. Trans-acting factors controlling developmental expression

Two transcriptional activators, GBF (Schintzler *et al.*, 1994) and STAT (Kawata *et al.*, 1997), that regulate developmental genes of *D. discoideum* have been characterized. GBF binds with CAEs while STAT has been shown to interact with an A/T-rich element of the prestalk gene *ecmB*. Electrophoretic mobility shift assays showed that box A binds to a factor that is present in the nuclear fraction. This factor

appears between 6 h and 12 h of development (Figure 24), a profile consistent with the timing of the resurgence of *rnrB* transcript (Tsang *et al.*, 1996). The binding of box A to this factor was not affected by the addition of excess CAE-1 (Figure 23) or an oligonucleotide containing a STAT-binding site (data not shown). These data suggest that box A interacts with a nuclear factor that has not yet been characterized. Moreover, results from electrophoretic mobility shift assays performed with mutant versions of box A show that the GCGC site near the 3' end of box A is important for binding to that factor. This indicates that this developmentally regulated factor is different from the factor responsible for repression of *rnrB* expression in prestalk cells, as mutations in the GG dinucleotide site near the 5' end of box A interfered with this activity (see Figure 20). The presence of other activating sequences in the -Δ450/A2 construct may explain why this mutant exhibits normal staining pattern in late developing cells.

Box B interacts with a factor that can be detected in the cytosolic fraction during growth and development (Figure 27). This factor is detected in the nuclear fraction of developing cells but not vegetative cells (Figure 28), suggesting that the factor migrates to the nucleus or is modified in the nucleus during development. Box B contains G/T repeats that resemble half of the consensus GBF-binding site. Results from competition assays suggest that box B interacts weakly with GBF (Figure 22). This observation implies that GBF does not have a major role in the regulation of *rnrB* expression (Bonfils *et al.*, 1999).

4.4. Box A may be a general repressor

We have found that box A of the *rnrB* promoter is involved in all aspects of the regulation of the expression of this gene: cell cycle control, DNA-damage control and

developmental control. We have measured the β-galactosidase activity directed by constructs bearing only box A, and two different mutant versions of box A, A1 and A2, in vegetative cells. Mutations in the A1 site near the 5' end of box A caused a 3-10-fold increase in promoter activity, suggesting that this site is bound by a transcriptional repressor (Figure 5). Mutations in the same site in the context of the full-length promoter caused abnormal expression in prestalk cells of developing cells (Figure 20), again suggesting that prestalk cells repress expression of *rnrB* by this same sequence. Finally, box A is required for response to MMS (Figure 13). In other species, increased gene expression in response to DNA-damaging agents is often mediated by removal of repression (see Section 1.2.4). It is possible that all these responses are mediated by the same factor.

5. CONCLUSIONS

1. The *rnrB* gene displays periodic expression during the cell cycle.
2. A DNA fragment comprising box A can confer cell-cycle regulation.
3. The *rnrB* gene is up-regulated following treatment with genotoxic agents.
4. The *rnrB* gene is up-regulated in the presence of hydroxyurea in vegetative cells but not in early developing cells.
5. The DNA damage reponse is independent of the developmental control of *rnrB* expression.
6. The DNA damage response appears to be modulated by factors present in the cell that become activated very rapidly after treatment.
7. The *rnrB* promoter can be used to control the expression of exogenous genes.
8. Different *cis*-regulatory elements are involved in the response to different genotoxins.
9. A genomic deletion of the DNA-damage reponse elements increases the sensitivity of *Dictyostelium* cells to DNA-damaging agents as well as cell-cycle arrest following treatment with mutagens.
10. Boxes A and B are independently able to confer developmentally-regulated, cell-type-specific gene expression.
11. A genomic deletion of box A and box B of *rnrB* causes no developmental defect.
12. We have detected the presence of a developmentally-regulated box A-binding activity that could be involved in postaggregative cells and/or prespore-specific expression of the *rnrB* gene.
13. The sequence GCGC near the 3' end of box A appears important for expression in prespore cells in late development and to bind to the box A binding factor.

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