Regulation of ribonucleotide reductases in growth and development in the social amoebae *Dictyostelium discoideum*

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Abstract

Regulation of ribonucleotide reductases in growth and development in the social amoebae *Dictyostelium discoideum*

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Growth and development are mutually exclusive phases of the life cycle of the cellular slime mould *Dictyostelium discoideum*. The amoebae grow as single cells and divide by binary fission until deprived of nutrients. Starvation forces the cells to initiate development where they organize into multicellular structures, each consisting of a spore head supported by a stalk. Prior to terminal differentiation, the prespore cells undergo a round of DNA synthesis, but it is not known whether this DNA synthesis takes place in the nucleus or the mitochondria.

Ribonucleotide reductase (RNR) converts ribonucleotides to deoxyribonucleotides and is the rate-limiting step of DNA synthesis. The expression of this enzyme has been temporally and spatially correlated with DNA synthesis and the expression may therefore be used as a marker for DNA synthesis. *Dictyostelium* is unusual among eukaryotes in that it harbours genes encoding two classes of RNR enzymes.

Using a short-lived β-galactosidase as a reporter, we have found the two subunits of the Class I RNR to be mainly expressed in prespore cells. The subcellular localisation of the RNRs was examined during growth and early development using fluorescent fusion proteins. While the subunits of the Class I RNR are spatially separated, the Class

II enzyme resides in the cytoplasm. Using gene replacement strategy to examine functions, we have shown that the gene encoding the Class II RNR is not essential for *Dictyostelium* growth and development.

For LOL

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act6p: actin 6 promoter

act15p: actin 15 promoter

Amp: ampicillin resistance

AX: axenic

β-gal: β-galactosidase

bp: base pairs

bsR: blasticidin S resistance

BrdU: 5-bromo-2-deoxyuridine

C: Celsius

Ca: calcium

CAE: C/A rich element

CDK: cyclin dependent kinase

DAPI: 4,6-Diamidino-2-phenylindole

Dicty: *Dictyostelium discoideum*

DIF: differentiation inducing factor

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleic triphosphate

EtOH: ethanol

FITC: fluorescein isothiocyanate

G1: growth phase 1

G2: growth phase 2

Gen: geneticin (neomycin) resistance

GFP: green fluorescent protein

GR: gene replacement

K. aerogenesis: Klebsiella aerogenesis

kb: kilo base

KO: knockout

Loflo: low fluorescent medium

M: mitosis

μg: microgram (10^{-6} g)

μM: micromolar (10^{-6} M)

mM: millimolar (10^{-3} M)

min: minute

ndr: nucleotide reductase

Neo: geneticin (neomycin) resistance

PCR: polymerase chain reaction

R1: ribonucleotide reductase Class I large subunit

R2: ribonucleotide reductase Class I small subunit

Rb: retinoblastoma

rbl: retinoblastoma-like protein

RFP: red fluorescent protein

RNR: ribonucleotide reductase

S: synthesis

WT: wild type

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

1 Introduction

Ribonucleotide reductases (RNRs) catalyze the rate-limiting step in DNA synthesis and nearly all organisms have at least one active enzyme. Owing to the importance of this enzyme in DNA synthesis, its encoding genes are strictly regulated on several levels. Errors in RNR regulation can have serious consequences to the cell such as DNA damage, cell cycle arrest and tumour promotion

Dictyostelium discoideum lies on the evolutionary border between unicellular and multicellular organisms. It spends parts of its life cycle as single cells, but enters development upon starvation to form multicellular structures. The facultative metazoan nature of its life cycle has made *Dictyostelium* a model organism for studying developmental processes. In addition, *Dictyostelium* is one of three eukaryotes known to date that has two RNR enzymes from two different classes (Lundin, 2005).

1.1 The cell cycle and its control of gene expression

The eukaryotic cell cycle usually consists of four phases: M-phase when mitosis and cytokinesis take place, S-phase when DNA is replicated and the two gap phases G1 and G2 that separate the M- and S-phases. Progression through the cell cycle is mediated by cyclins and cyclin-dependent protein kinases, cdks. The activity and substrate specificity of the cdks are regulated by cyclins (Murray, 1993). Hence different cdks are activated by the presence of their conjugate cyclins at different phase transitions of the cell cycle.

The G1 to S transition of the cell cycle is commonly under strict control. At this point, cell cycle progression is coordinated with the DNA replication machinery, information of nutrient availability and information from surrounding cells. When entry

to S-phase is assured, the cell will usually continue through the entire cell cycle. The G1/S transition point has been named "R" (Restriction point) in mammals and "START" in yeast. Cells arrested in the cell cycle are usually arrested at this point (Whitaker, 1990). In addition, some organisms have a major checkpoint that regulates the exit from G2 and entry to mitosis (Murray, 1993).

Entry to S-phase is accompanied by expression of genes necessary for DNA synthesis, many of which are regulated by the cell cycle repressor, pRb. This is the gene product of the retinoblastoma susceptibility gene and it binds and inactivates several transcription factors including E2F during most of the cell cycle. In mid/late G1, pRb is phosphorylated by cdks and is consequently inactivated. The inactivation of pRb enables E2F to mediate transcription of S-phase genes (DeGregori, 1995; Nakagami, 1999). The pRb inactivation followed by expression of S-phase genes and cell cycle progression is seemingly a universal control system common to eukaryotes (Xie, 1996) with the exception of yeast (reviewed by Gutierrez, 2005). If pRb is not properly regulated, it can lead to premature entry to S-phase. This is an effect found in many types of cancer (Classon, 2002).

Dictyostelium differs from many other eukaryotic systems in its lack of a G1-phase; after mitosis *Dictyostelium* cells proceeds directly to S-phase (Weijer, 1984a). Both M- and S-phase are relatively short in duration and make up approximately 1 hour of an 8-hour *Dictyostelium* cell cycle (Weijer, 1984a). How the cell cycle and G1-phase genes are regulated is not well understood. S-phase genes have been shown to be transcribed during M-phase as well as in the "phase of transition" that is in early- to mid-G2 of the *Dictyostelium* cell cycle (MacWilliams, 2001). This "phase of transition" is not a clearly

defined point, but a period of intracellular changes often seen at the G1/S transition in more understood systems. These changes involve a transient increase in intracellular pH and Ca^{2+} level (Aerts, 1985; Azhar, 2001), which has been thought to play a role in the cell cycle and especially S-phase regulation (Aerts, 1985; Whitaker, 1990).

Dictyostelium has a gene that encodes a pRb orthologue, *rblA* (retinoblastoma-like), and a single gene encoding E2F. An *rblA*-null strain has been obtained and the removal of this gene does not lead to any obvious changes in the *Dictyostelium* cell cycle (MacWilliams, 2006). In comparison, knocking out this gene in a mammalian cell line leads to premature entry to S-phase (reviewed by Classon, 2002). Over-expression of *rblA*, however, leads to an pronounced G1-phase even though *Dictyostelium* cells usually do not display this phase of the cell cycle (MacWilliams, 2006).

1.2 Development and tissue specific DNA synthesis in *Dictyostelium*

As long as food is present, *Dictyostelium* cells divide and grow as single cells, but upon starvation, the cells enter development and up to 10^5 cells aggregate and develop into a multicellular structure consisting of two broad cell types: spore cells and stalk cells. The developmental cycle takes 24 hours and is marked by two salient morphological stages: 8 hours following starvation the cells form aggregates in response to cAMP signals and at about 16 hours the cells are organized in finger-shaped structures called migrating slugs. The slug is differentiated along the anterior/posterior axis, where prestalk cells occupy the anterior one-quarter of the structure (reviewed by Loomis, 1996).

At approximately 18 hours of development the slugs stop migrating and the anterior cells force their way through the tip. The reverse fountain-like movement of the anterior cells help the posterior cells to propel upward, resulting in a ball of spores (previously the posterior part of the slug) resting on a stalk of vacuolized, dead cells (previously the anterior or the slug) (reviewed by Gaudet, 2008; Loomis, 1996).

During development, there is an approximate doubling of the cell number. The increase occurs in two distinct parts of the developmental cycle; one during the first 6 hours of development and one in late development (Zada-Hames, 1978). The first round of cell division is attributed to completion of mitosis by cells that are in or close to the M and S-phases by the onset of starvation. It is generally assumed that at 8 hours of development, all the cells are in G2 of the cell cycle (Weijer, 1984b; Zimmerman, 1993). The second round occurs in prespore cells and peaks at 18 hours of development. At the same time, the prespore cells undergo a wave of DNA synthesis. Early experiments, using incorporation of ^{3}H-thymidine or BrdU as a marker for DNA synthesis concluded that the DNA synthesis was fuelling nuclear DNA replication in the prespore cells (Durston, 1978; Zada-Hames, 1978; Zimmerman, 1993). The increase in cell number was attributed to an M-phase followed by DNA replication and that the cells were assumed to undergo terminal differentiation as G2 cells (Zada-Haimes, 1978). However, Chen *et al.* (2004) reached a different conclusion when they observed that BrdU was only found in the cytosolic fraction of developing cells. They stated that the cells would replicate their mitochondrial DNA before going through mitosis and then differentiate into spores as G1 cells (Chen, 2004; Shaulsky, 1995). In contrast to the finding of Chen *et al.* (2004), direct live imaging of cells during cell cycle and development using a Green Fluorescent

Protein fused to a replication factor, PCNA, revealed that the S phase machinery is active in the nucleus during late development, and this finding led to the conclusion that *both* nuclear and mitochondrial DNA was replicated in late development (Muramoto, 2008). The same conclusion was reached by Deering and Michrina (Deering, 1982a), and both groups concluded that the spore cells are in G2.

There are limitations to each of the methods used to determine where DNA replication occurs in late development, including whether the uptake and incorporation of precursors to the DNA is stable or not: the degree of incorporation of different labelled precursors will vary between mitochondrial and nuclear DNA at different times of development (Deering, 1982b). Due to the conflicting results, it is still disputed whether the prespore cells enter terminal differentiation as G1 or G2 cells.

1.2.1 The RblA protein and its involvement in *Dictyostelium* development

The retinoblastoma susceptibility gene product, pRb, is not only involved in cell cycle control but has been found to play a role in numerous activities in the cell such as regulation of differentiation and apoptosis (reviewed by Classon, 2002). In mammals, its inactivation in differentiated tissue is correlated with many forms of cancer while Rb-/- mice die before birth with defects in nervous system, blood cells, skeleton and muscle tissue (reviewed by Classon, 2002). The pRb protein has many phosphorylation sites which generates the potential of several different binding partners (reviewed by Taya, 1997) and it is believed to bind and interact with hundreds of different partners depending on context (Classon, 2002). In *Dictyostelium, rblA* is expressed in prespore cells during development with an expression peak between 12 and 16 hours. Interestingly, knocking

out *rblA* in *Dictyostelium* leads to a shorter developmental cycle in contrast to the impairment seen in mammalian systems (MacWilliams, 2006).

When *rblA*-null cells are mixed with wild type cells and starved, *rblA*-null cells show a preference for the stalk pathway and an increased sensitivity for the stalk inducer Differentiation Inducing Factor, DIF-1 (MacWilliams, 2006). The RblA protein also seems to repress transcription of S-phase genes; expression of these genes has been shown to increase in the knockout strain, both in developing and growing cells (K. Straβer, personal communication).

1.2.2 Cell cycle position upon starvation is correlated to cell fate

Cell fate in *Dictyostelium* cells is influenced by where in the cell cycle the cells are at the time of starvation. Cells in S-, M- and early G2-phases are predisposed to become stalk cells, while cells in mid- and late-G2 are more likely to become spore cells (Azhar, 2001; Gomer, 1996; Gomer, 1987; McDonald, 1984; Ohmori, 1987; Weijer, 1984a; Zimmerman, 1993).

There are two theories explaining the mechanism behind cell type choice: one postulates that the cells continue cell cycle progression when starved until they reach a Putative Shift point, "PS-point", towards the end of G2, when the cells start expressing developmental genes. Cells reaching the PS-point early are favoured to enter the spore pathway (reviewed by Maeda, 1993; Maeda, 1989).

Alternatively, it has been postulated that cells retain their cell cycle position when they enter development and that cell signals already present within the individual cell will steer it towards one developmental fate or the other (MacWilliams, 2001). The

observation of a "phase of transition" in early- to mid-G2 coinciding with the switch in cell fate choice supports this theory. In mid-G2 several intracellular changes occur, such as an increase in the intracellular pH and decrease in the intracellular calcium content, both features of cells in the spore pathway (MacWilliams, 2001). Other intrinsic factors also play a role in determining cell fate such as nutrient history: cells grown on high glucose will more commonly become spore cells when mixed with cells grown in medium with lower glucose concentration (Weijer, 1984b).

1.3 Ribonucleotide reductases

Ribonucleotide reductases (RNR) catalyze the reduction of ribonucleotides to deoxyribonucleotides, the first step in *de novo* DNA synthesis (reviewed by Jordan, 1998; Nordlund, 2006). Its importance in the cell cycle is reflected in its role in DNA synthesis and repair. Failure in controlling this enzyme can lead to cell abnormalities such as DNA damage and cell death. The RNRs are up-regulated in many forms of cancer and they are the target of several kinds of cancer treatments (Shao, 2006).

Ribonucleotide reduction catalyzed by RNRs involves the generation of a free radical within the enzyme and the enzymes are classified into three main classes based on how this radical is generated. The Class I enzymes are tetramers, usually composed of two homodimers. The two homodimers are commonly referred to as the large subunit, or R1, and the small subunit, R2. The large subunit contains the catalytically active site, it binds the substrate and can be allosterically regulated by dATP. R2 is essential for enzyme activity and generates a tyrosyl radical whose stability is dependent on an iron centre within the subunit. This radical is then shuttled to a cysteine residue in the large

subunit upon enzyme activity (Eklund, 2001). The C-termini of R2 has also been found to be essential for stability of the holoenzyme (Eklund, 2001). Class I RNRs are dependent on molecular oxygen for proper function and are found in nearly all eukaryotes and several aerobic and facultatively aerobic microorganisms. The substrate of Class I RNRs is ribonucleotide diphosphates (reviewed by Nordlund, 2006).

The Class II enzymes function independently of oxygen and generate a cysteine radical with the aid from adenosyl cobalamin (vitamin B_{12}). Class II enzymes are usually found in prokaryotes but a few eukaryotes also harbour this enzyme either as the only one, or in addition to a Class I enzyme (Lundin, 2005). In eukaryotes it is a monomer and it reduces ribonucleotide triphosphates (Torrents, 2005). Class III enzymes are inhibited by oxygen and only found in prokaryotes.

Genome sequence analysis revealed that *Dictyostelium* is one of three known eukaryotes with more than one class of RNR (Lundin, 2005). It has a Class I enzyme, encoded by the genes *rnrA* (large subunit) and *rnrB* (small subunit) and a Class II enzyme encoded by the *ndrJ* gene. In addition it has a second gene with significant similarity to the gene encoding the small Class I subunit, *rnrB2* (dictybase gene ID: DDB_G0291764). This gene seems to encode an incomplete protein that lacks approximately 25% of the C-terminal in addition to several amino acids known to be necessary for catalytic activity and therefore may encode a pseudogene (Fey, 2009).

Having several functional RNR classes is uncommon among eukaryotes (Lundin, 2005), but in prokaryotes all combinations of the different classes can be found within one organism (Torrents, 2002). Some prokaryotes might use their range of enzymes under different oxygen conditions, but species with a Class I and a Class II enzyme,

transcriptionally active at the same time, have been found. How these enzymes compete with each other and why some organisms have kept them both during the course of evolution is not known, but their use of different substrate or small differences in allosteric regulation could provide the organism with an advantage (Jordan, 1999). In contrast, mammals and yeast have an additional gene encoding for the RNR small subunit or the RNR large subunit, respectively. Transcription of the additional genes are induced under genotoxic stress (Elledge, 1993; Lozano, 2000).

1.3.1 Transcriptional regulation of ribonucleotide reductases

An unbalanced pool of dNTPs can have serious consequences for the cell. Low dNTP concentration can inhibit DNA synthesis and repair, and the cell can go into cell cycle arrest. In multicellular organisms, abnormally high dNTP concentrations can promote cancer and metastasis (Shao, 2006). Because of their importance, transcription of the RNR genes is a strictly regulated event initiated as a response to either S-phase entry or DNA damage. In *Dictyostelium*, the RNR genes has also been shown to be expressed in late development (Bonfils, 1999).

In most organisms, transcription of RNR genes is initiated in mid- to late G1-phase and accumulates as S-phase progresses (Björklund, 1992; Elledge, 1993). *Dictyostelium* does not have a G1-phase and studies of *rnrB* have revealed that this gene is transcribed twice during a cell cycle: once in late G2-phase and once in mid-G2, correlating to the "phase of transition" (MacWilliams, 2001). The latter is thought to feed mitochondrial DNA replication.

During *Dictyostelium* development, the RNR genes *rnrA, rnrB* and *ndrJ* are transcribed with an increased expression in late development that peaks at 18 hours (M.

Crona, personal communication). This corresponds to the wave of DNA synthesis in prespore cells during development, and *rnrB* expression has indeed been found to be prespore specific (Tsang, 1996). Both *rnrA* and *rnrB* are transcribed at a higher level than *ndrJ* (M. Crona and K. Straβer, personal communication).

The only RNR promoter studied in *Dictyostelium* is the one of *rnrB*. This promoter has three GC rich elements essential for promoter activity (Bonfils, 1999) where *trans*-factors bind in different combinations upon DNA damage (Gaudet, 1999) and development (Bonfils, 1999; Gaudet, 1999). An A/T rich element close to the transcriptional start site is enough to drive expression during vegetative growth (Bonfils, 1999). Such A/T rich elements are common in cell cycle regulated genes (Powell-Coffman, 1994). A fourth G/C rich element is thought to repress promoter activity and release from this inhibition is essential for transcriptional induction upon DNA damage. Which *trans*-factors bind to these promoters are not known (Bonfils, 1999).

Knocking out the four G/C rich elements in the *rnrB* promoter inhibits *rnrB* transcription during development and increases cell sensitivity to DNA damaging agents. Even though no *rnrB* expression was reported in these cells, they developed normally (Gaudet, 2001).

An indication as to one of the transcriptional regulators of the three RNR genes has been found: microarray and RNA sequencing of *rblA*-null cells shows that the expression of *rnrA* and *rnrB* is up-regulated several-fold compared to wild type cells. Interestingly, *ndrJ* seems to be slightly down-regulated in this strain (K. Straβer, personal communication).

The retinoblastoma gene product, pRB, regulates RNR transcription in several systems, both through the E2F transcription factor and other unknown factors (reviewed by Taya, 1997). The regulation of RNR by E2F is complex and not very well understood; for instance, E2F binding sites are found in the RNR promoters of tobacco and in *Arabidopsis*. The function of the E2F binding sites are dual, they induce gene transcription during S-phase but represses RNR transcription during the remainder of the cell cycle (Chabouté, 2000). In human and mouse, E2F inhibits transcription from both RNR subunits during normal cell cycle. Its effect on the promoter during S-phase is not known and direct binding between E2F and the promoter has only been shown for the small subunit (Angus, 2002; Chabes, 2004; Thelander, 2007).

1.3.2 Spatial regulation of ribonucleotide reductases

One way of regulating protein activity in eukaryotes is to separate the subunits of a protein into different compartments (Weis, 2003). Spatial separation is used in regulation of several proteins involved in cell cycle regulation and proteins that are inactive in parts of the cell cycle (Takizawa, 2000).

The subunits of RNR have been localized in yeast, human and plants. In *Saccharomyces cerevisiae*, the RNR large subunit, R1, is localized to the cytoplasm and the small subunit resides in the nucleus. Upon DNA damage or entry to S-phase, the small subunit is relocalized to the cytoplasm where it associates with R1 to form an active holoenzyme (Yao, 2003; Zhang, 2005). In human cells, localisation of the subunits depends on the need of the cell: the large subunit resides in the cytoplasm during the cell cycle together with the small subunit, R2, where they associate at the entry of S-phase (Larsson, 1969; Leeds, 1985). A second small subunit p53R2 is found in the nucleus, and

the large subunit is translocated to the nucleus upon DNA damage (Liu, 2005; Lozano, 2000; Xue, 2003). p53R2 seems to be the active RNR small subunit in quiescent cells and is found to associate with R1 when DNA is damaged and it is also present in high levels in the mitochondria (reviewed by Thelander, 2007). Similar to mammals, the tobacco R1 subunit migrates from the cytoplasm to the nucleus upon UV-irradiation (Lincker, 2004). There seems to be a general consensus that during normal cell growth, dNTPs are synthesized by a ribonucleotide reductase enzyme that is residing in the cytoplasm and the transport of dNTPs into the nucleus relies on diffusion. The response to DNA damage might shift the location of ribonucleotide reduction (Leeds, 1985).

1.4 Mitochondrial DNA replication

The mitochondria are far more than the ATP factory of the cell, and they are of importance in growth, differentiation, cell type determination and apoptosis. A low number of mitochondria within the cell or imbalance in the intra-mitochondrial dNTP pools leads to several severe diseases in human (reviewed by Mathews, 2007). Mitochondria are crucial for proper transition from growth to differentiation, cell type determination and pattern formation in developing *Dictyostelium* cells (reviewed by Maeda, 2005). In addition they are found to guide phototaxis and chemotaxis in the migrating slug and apoptosis in the prestalk cells (reviewed by Barth, 2007). Removing most of the mitochondrial DNA from developing *Dictyostelium* cells has been shown to impair spore formation in spite of normal respiration levels (Chida, 2004).

The pools of dNTPs are interchangeable between cytoplasm and mitochondria, but they are of slightly different nucleotide composition (Rampazzo, 2004). Several

models for nucleotide import and salvage pathways within the mitochondria have been proposed in addition to the detection of RNR activity (reviewed by Mathews, 2007). The RNR activity is higher in mitochondria than in cytoplasm of both liver and HeLa cells and it is not inhibited by dATP which usually acts like an allosteric inhibitor of the Class I RNR (Young, 1994).

In *Dictyostelium,* mitochondrial DNA can make up as much as 30% of the total cellular DNA but little is known about mitochondrial DNA replication, except that it most likely takes place during mid-G2 phase of the cell cycle (Weijer, 1984a) and that there is mitochondrial DNA replication in prespore cells during development (Shaulsky, 1995). *Dictyostelium* spore cells are also derived from mid/late G2 cells, cells from the part of the cell cycle with the highest mitochondrial number. Improved insight into the function and localisation of the RNR enzyme might enhance our knowledge about the DNA replication of this important organelle.

1.5 Rationale behind the experiments

Disruption of a gene can both give knowledge as to whether it is essential and, if a phenotype is obtained, to the potential function of the protein. In cases where several proteins that carry out the same function are present, a knockout or a combination of knockouts could elucidate whether the proteins are able to compensate for each other. *Dictyostelium* is one of a few eukaryotes that have several genes encoding more than one RNR and the reason for having several enzymes carrying out the same function and whether these enzymes compete or complete each other's functions are not known. To

examine this question we wanted to generate a set of gene replacement strains where one or several RNRs were functionally knocked out.

The expression of *rnrB* is prespore specific during development in *Dictyostelium.* There is no similarity between the *rnrB* promoter and the promoters of the other RNR genes. Whether these genes are also expressed in a cell-type specific manner is not known. The RblA protein represses transcription from these promoters, but how this repression functions has not been examined. To examine these questions we created a set of plasmids where a β-galactosidase reporter gene is placed under control of the different RNR promoters. These plasmids were transformed into both wild type *Dictyostelium* cells and the *rblA*-null strain so that the cell type specific localization of RNR transcription during development could thus be assessed.

In yeast and mammals, ribonucleotide reductase activity is localized in the cytoplasm during normal growth, but the localization pattern changes when the cells are subjected to DNA damage. In mammals, RNR activity has also been found in the mitochondria. The site or sites of ribonucleotide reduction is not known in *Dictyostelium.* To examine this question, a set of fluorescent fusion proteins were created to examine localization of the various RNR proteins *in vivo.* It is also possible to subject the cells to stressors such as UV light and a potential change in localization could be detected.

During *Dictyostelium* development, DNA synthesis takes place, but whether this synthesis feeds nuclear or mitochondrial DNA replication is not known. Moreover, whether the *Dictyostelium* prespore cells are in G1 or G2 when they undergo terminal differentiation is still disputed. Using fluorescent fusion proteins to localize the ribonucleotide reductase activity in late development where DNA synthesis takes place,

could give an indication as to whether it is nuclear or mitochondrial DNA that is being synthesised.

2 Materials and methods

2.1 General techniques

2.1.1 Strains

The axenic strains AX2 ("wild type") and its derivative *rblA*-null (MacWilliams, 2006) were used in this study. The latter strain is a functional null mutant for the *rblA* gene and was constructed by insertion of a blasticidin cassette into the *rblA* coding region. *rblA*-null cells show a somewhat precocious development and a preference to the stalk pathway compared to AX2. The *rblA*-null strain was provided by H. MacWilliams.

2.1.2 Growth and developmental conditions

Dictyostelium cells were grown at 22°C on SM agar with a lawn of *Klebsiella aerogenesis* (Sussman, 1966) or axenically in HL-5 medium (Watts, 1970). Axenic cells were either grown attached to the bottom of plastic Petri dishes or shaken at 150 rpm in Erlenmeyer flasks. The shaken cultures were kept at densities between $1x10^5$ cells/ml and $2x10^6$ cells/ml.

To initiate development, cells were harvested by centrifugation for 5 min at 4°C and 500g and washed twice in KK2 buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2). The cells were then spread onto moist nitrocellulose membranes at a density of $2x10^6$ cells per cm^2 (Sussman, 1987).

2.1.3 Isolation of genomic DNA

Genomic DNA was extracted from *D. discoideum* by first isolating the nuclei according to the method of Cocucci and Sussman (Cocucci, 1970). The DNA was then extracted according to Sambrook *et al.* (Sambrook, 1989).

When screening for gene replacement strains, a rapid DNA extraction protocol was used to obtain a crude DNA extract (Charette, 2004). This extract was used directly in PCR reactions.

2.1.4 Amplification of genes from *Dictyostelium* genomic DNA using PCR

All PCR reactions were carried out in the following manner: oligonucleotide primers were suspended to a concentration of 50 μM in ddH_2O and optimal annealing temperature for amplification was determined by pilot PCR reactions using varying annealing temperatures. Each reaction contained 0.1 μM of each primer, 0.2 mM dNTP mix, 0.5 μg genomic DNA, 5 units of *Taq* and 1 unit of *Pfu* DNA polymerases in 1xPCR buffer (20 mM Tric-HCl, 1.5 mM $MgCl_2$, 23 mM KCl, 0.05% Tween20, 100 μg/ml BSA, pH 9.5). Total volume was 100 μl.

The amplification was carried out using a thermal cycler and initiated by a 5 minute long denaturation at 94°C, followed by a two-step program. The first step was set to 1 minute denaturation, 2 minutes annealing at a gradient from 45°C to 55°C, and 2 to 4 minutes extension. The extension time was determined according to expected product length (1.5 min per kb). This step was repeated 10 times. The annealing temperature was then increased to a gradient from 50°C to 65°C for the second step and the cycle was run

an additional 20 times. Three percents of the product were analyzed on a 1.0% agarose gel cast in 0.5% TAE (40mM Tris-acetate, 1mM EDTA, pH 8.0).

The fragments obtained in the gradient PCR was used as a guide for determining optimal temperature settings for each primer pair and four to seven additional reactions were run at the optimal temperature (see Table 3 for specific settings depending on primer pair). These products were analyzed on an 0.7% agarose gel and the fragments were purified using either a QIAquick PCR Purification Kit from QIAGEN (cat. no 28104) or phenol:chloroform:isoamyl separation (Sambrook, 1989). The fragment was then cut using restriction enzymes followed by either band purification from a 0.7% agarose gel using a QIAquick Gel Extraction Kit from QIAGEN (cat. no. 28704) or inactivation of the restriction enzymes at 80°C for 20 min. The fragments were ligated into linearized plasmid using T4 DNA ligase from Invitrogen at 14°C for 12 hours.

2.1.5 Amplification and screening of plasmids

Ligated plasmids were transformed into *Escherichia coli* strain XL1-Blue (endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+) by electroporation. Cells containing the plasmid were selected for by plating on LB plates (1% Bactopeptone, 0.5% yeast extract, 1% NaCl, 1.5% Bactoagar) with ampicillin at a concentration of 60 μg/ml. Plasmids were extracted from the transformants using alkaline lysis method (Sambrook, 1989). Individual plasmids were screened for using restriction endonuclease digestion.

2.1.6 Transformation of *Dictyostelium*

Linearized vectors were electroporated into *D. discoideum* cells using the method of Schlatterer *et al.* (Schlatterer, 1992). Transformed cells were diluted in HL-5 medium and aliquoted to three 96-well plates. The cells were allowed to recover overnight (16-24 hours) before antibiotic (blasticidin) was added to a final concentration of 10 μg/ml. Medium and antibiotic were changed every 2-3 days for two weeks while colonies were allowed to form.

Expression vectors were transformed using the calcium phosphate method described by Nellen and Early (Nellen, 1984) and reviewed by Gaudet *et al.* (Gaudet, 2007). The transformed cells were allowed to recover at 22°C overnight before the appropriate antibiotics were added (geneticin at 20 μg/ml or blasticidin at 10 μg/ml). Medium was changed every 2-3 days for two weeks until colonies were visible.

Before any experiments were pursued, the cells were grown in medium without antibiotics for at least 24 hours.

2.2 Gene disruption by homologous recombination

2.2.1 Construction of gene replacements plasmids

With the objective of disrupting the four RNR encoding genes, four vectors were constructed. The upstream (5') and downstream (3') flanking regions of each gene were amplified separately and cloned into the plasmid pLPBLP sequentially. The pLPBLP was obtained from dictybase.org and is designed for gene disruption by having two multiple cloning sites flanking a blasticidin (bsR) cassette (Faix, 2004). Two loxP sites flanking

the antibiotic cassette enable for repeated use of the resistance gene (see Figure 1). Four plasmids were constructed in this manner, the rnrA-pLPBLP (Figure 2), rnrB-pLPBLP (Figure 3), ndrJ-pLPBLP (Figure 4:) and rnrB2-pLPBLP (Figure 5). Primers used in these reactions are listed in Table 1.

2.2.2 Disruption of genes

The plasmid rnrB-pLPBLP was linearized using PvuII or in a double digest with ClaI/SpeI. The linearized plasmid was gel purified using the QIAquick Gel Extraction Kit from QIAGEN (cat. no. 28704). Twenty micrograms of linearized product were electroporated into exponentially growing AX2 cells (Schlatterer, 1992). A total of 20 colonies from five electroporation attempts were screened for gene replacement using PCR. Both crude extract and purified DNA was used as templates in reactions where one primer bound upstream of the recombining arms and the other inside the disrupted region (the same localization relative to the gene as primers used to screen for a *ndrJ* gene replacement strain, see Figure 7).

The plasmid ndrJ-pLPBLP was linearized using either PvuII or a combination of ClaI and BamHI. Linearized plasmid was purified as above and 30 μg were transformed into AX2 cells using electroporation. A total of 91 possible gene replacement strains were screened with primers binding both external and internal to the disruption site. Crude DNA extracts were used as templates. Clones positive for gene replacement were purified by plating for single colonies on *K. aerogenesis* lawns on SM plates before the DNA was extracted using the method by Cocucci and Sussman (Cocucci, 1970). Purified clones were screened a second time using PCR. The primers would direct amplification across

the region of potential gene replacement to obtain a shift in product size (see Figure 7 for binding primer binding sites).

The plasmid rnrB2-pLPBLP was linearized using PvuII and cleaned using phenol:chloroform:isoamyl separation (Sambrook, 1989). Thirty micrograms of linearized plasmid were transformed into exponentially growing AX2 using electroporation. Five colonies were screened using PCR with a combination of externally and internally binding primers. Crude DNA extracts were used as template.

No gene disruption was attempted using the rnrA-pLPBLP plasmid.

2.2.3 Growth of *ndrJ* disrupted mutants

Two individual strains with disrupted *ndrJ* were diluted to $1x10^5$ cells per ml in 50 ml HL-5 medium. The cells were cultured in 250 ml flasks at 150 rpm at room temp. Approximately every 2 hours, 10 µl cells were withdrawn and counted using a haemocytometer. Each strain was counted at least twice, depending on the variation in the counts. The controls used were AX2 and AX2 with a randomly integrated bsR cassette. Cells were also diluted and plated on SM plates with *K. aerogenesis* as a viability assay.

2.3 *In situ* localization of reporter gene products

2.3.1 Construction of lacZ expression vectors

Three plasmids were constructed with the objective of determining the location of promoter activity in the developmental structures. A β-galactosidase with a short half-life was used as a reporter and expressed under the various RNR promoters.

The backbone used for this study was the plasmid rnrB-ile-αgal where the ile-αgal is a β-galactosidase with increased activity, but with a protein half-life of only 30 minutes (see Figure 8 for physical map) (MacWilliams, 2001). The 450 bp *rnrB* promoter already present in the plasmid was excised using XbaI and BglII and replaced with the promoter of interest. Three additional reporter plasmids were constructed in this manner: the rnrA-ile-αgal (see Figure 9), ndrJ-ile-αgal (see Figure 10) and rnrB2-ile-αgal (see Figure 11) (see Table 2 for list of primers used to amplify these promoters).

2.3.2 Transformation and screening for cells producing β-galactosidase

Thirty micrograms of each of the four expression vectors were transformed into both AX2 and *rblA*-null cells using the $CaPO_4$ method by Nellen (Nellen, 1984). Transformed cells were selected for by the addition of geneticin at 20 μg/ml. When colonies started to form, the cells were diluted and plated to obtain single colonies on KK2 plates (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, 1.5% agarose, pH 6.2) with a lawn of *K. aerogenesis*. When colonies appeared on the plates, they were lifted onto nitrocellulose filters and screened for β-galactosidase activity in the following manner: filters containing colonies were placed at -20°C for a few minutes before they were fixed onto the filters using

glutaraldehyde solution (0.05% glutaraldehyde and 0.04% Tween20 in Z-buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCP, 1mM $MgCl_2$, pH 7.0)). The filters were then washed and incubated in 1 part Dingerman cocktail (5mM K3[Fe(CN6)], 5mM K4[Fe(CN6)], 1mM EGTA) (Dingerman, 1989) in 4 parts of Z-buffer and 1 mM X-gal in DMF. The reaction was continued until a blue colour appeared, approximately 1 hour, and the colour was fixed in 3% TCA before the filters were washed and dried. This histochemical procedure was adapted after the method described by Dingerman *et al.* (Dingerman, 1989).

Approximately 7 colonies from each of the rnrA-ile-αgal and rnrB-ile-αgal producing strains were picked and pooled for use in further experiments. Cells transformed with ndrJ-ile-αgal and rnrB2-ile-αgal were pooled and used without prior successful screening.

2.3.3 Histological localization

β-galactosidase producing cells were grown to a density of $1x10^6$ cells/ml and starved by washing in KK2 buffer, and plated on moist, pre-boiled nitrocellulose filters at a density of $2x10^6$ cells per cm^2. The cells were left to develop for 2 to 22 hours before they were fixed onto the filters with 0.05% glutaraldehyde (see above). The filters were then belly flopped into 0.1% glutaraldehyde before washing with Z-buffer. The filter pieces were incubated with X-gal for approximately one hour until a blue colour was visible on the structures. To preserve the colour, the filter pieces were fixed in 3% TCA, washed and dried.

The developing structures were observed using a Nikon SMZ1500 stereomicroscope using 10x magnification and images were captured using a SPOT Insight Colour digital camera with 1.92 million pixels.

2.4 Localization experiments

To localize the RNR proteins within the cell, a series of plasmids encoding fluorescent fusion proteins were constructed. The plasmids encode either a GFP or a RFP with a RNR on the N-terminus of the translated products. The expression is controlled under the strong promoter from the Actin 15 gene.

When the RNR genes were amplified to be cloned into the plasmids carrying a fluorescent protein, the dNTP concentration in the PCRs were adjusted to better accommodate the *Dictyostelium* genomic A/T:G/C ratio. The concentrations of dATP and dTTPs were increased to twice of that of dCTP and dGTP (0.075 mM per reaction versus 0.025 mM). All plasmids were sequenced (McGill University and Génome Québec Innovation Centre) before being transformed into AX2.

2.4.1 Construction of green fluorescent fusion proteins

The full-length genes *rnrA, rnrB* and *ndrJ* were PCR amplified (see Table 2 for primer overview) and cloned into the multiple cloning site of the plasmid pDXA-GFP2 (see Figure 12: for physical map of pDXA-GFP2) (Levi, 2000), obtained from dictybase.org. The plasmids constructed were called GFP2-rnrA (Figure 13), GFP2-rnrB (Figure 14) and GFP2-ndrJ (Figure 15).

2.4.2 Construction of red fluorescent fusion protein

The plasmid mRFPmars-in-pBsrH was obtained from A. Müller-Taubenberger and it contains a red fluorescent protein where the codon usage is optimized for expression in *Dictyostelium*, the mRFPmars (see Figure 16 for physical map) (Fischer, 2004). This plasmid contained the actin gene. This actin gene was excised using the restriction endonuclease EcoRI and the plasmid was ligated back together which resulted in removal of the actin gene.

The full length *rnrA* and *rnrB* was amplified using the same forward primers as used to construct the GFP2-rnrA and GFP2-rnrB, respectively, but the reverse primer was changed to accommodate the different endonuclease recognition sites in the mRFPmars-in-pBsrH plasmid (see Table 2). The plasmids constructed were named mRFPmars-rnrA (Figure 17) and mRFPmars-rnrB (Figure 18).

2.4.3 Imaging of cells harbouring fluorescent fusion proteins

2.4.3.1 Sample preparation

Exponentially growing cells were incubated in Loflo+yeast medium (Low Fluorescent medium with yeast extract) (dictybase.org) for 1-4 hours before suspended in Loflo and dropped onto microscope slides submerged in Loflo. The cells were left to settle for approximately 15 minutes before imaging. Several fixation techniques were also attempted, either following incubation in Loflo or HL-5 medium. Cells were dropped onto microscope slides and fixed in methanol or 1% formaldehyde in methanol at -20°C. A coverslip was added immediately before imaging. Alternatively, the cells were left to settle on cover slides before fixation in ethanol (final concentration of 50% EtOH) (H.

MacWilliams, personal communication) at room temperature. Before imaging, the coverslips were dried and mounted on a microscope slide with diluted DAPI or ddH_2O.

To image cells in early development, exponentially growing cells were washed and suspended in KK2 buffer. The cells were transferred to Petri dishes and incubated for 4-5 hours to initiate development. These cells were then fixed onto cover slips using ethanol.

Fruiting bodies at 16-18 hours of development were obtained by washing and plating $2x10^6$ cells on 33 mm Petri dishes with 1ml of 1.5% agarose in KK2. Pieces of agarose were cut out and placed on a microscope slide. Immediately before imaging, a drop of immersion oil was placed onto the structures and cover slide was added. To dissociate the cells in developing structures, 10 - 20 slugs were picked up from the plates using a 27 gauge needle placed on a syringe and mixed with 100µl cellulase buffer (1% cellulase, 150 mM NaCl, 2 mM Na-EDTA, 20 mM KK2, pH 5.8) (K. Straβer, personal communication). The suspension was added onto microscope slides and imaged immediately.

2.4.3.2 Microscopy

Cells producing fluorescent fusion proteins were imaged using two different microscopes: A Zeiss Axioplan fluorescence microscope with the following filters: a GFP Filter Cube (#1031346, Exciter Filter: BP 470/40, Beam Splitter: FT 495, Barrier Filter: BP 525/30) and a dsRed Filter Cube (#1114462, Exciter Filter: BP 560/40, Beam Splitter: FT 585, Barrier Filter: BP 630/75). Nuclei stained with DAPI were visualized using a UV range filter cube (#487902, Exciter Filter: G 365, Beam Splitter: FT 395, Barrier Filter: LP 420). The microscope has a 50-watt mercury vapour lamp. Images were

taken using a SPOT Insight Colour digital camera at a resolution of 1.92 million pixels per frame.

The second microscope was a Leica microscope (model: DM6000B). GFP2 producing cells were observed using a 100x objective and an FITC filter cube (Semrock Brightline FITC-3540B, single band exciter: FF01-482/35-25, single band emitter: FF01-536/40-25). DAPI stained nuclei were imaged using a UV range filter cube (Semrock Brightline DAPI-5060B, single band exciter: FF01 377/50-25, single band emitter: FF02 477/60-25). The microscope has a 100-watt mercury lamp. Images were taken using a HAMAMATSU ORCA-ER digital camera (model: C4742-80-12AG) at a resolution of 1.37 million pixels per frame.

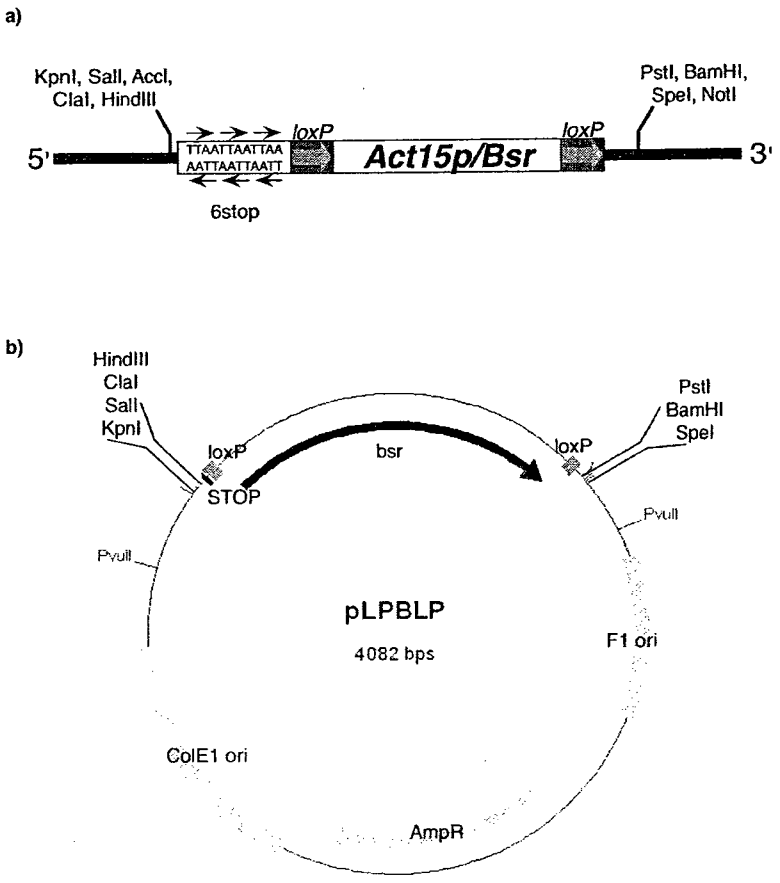
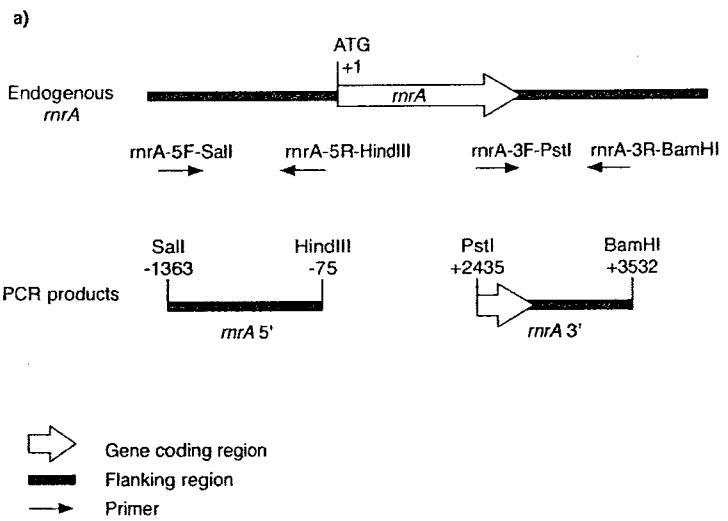


Figure 1: Physical map of the gene replacement vector pLPBLP

(a) Structural details of the Bsr cassette. The cassette consists of a Blasticdin resistance gene driven by the Actin 15 promoter. Upstream of the cassette are 6 stop codons, three on each strand covering all 6 possible reading frames. The stop codons introduce nonsense mutations that will prevent translation even if some of the coding region of the replaced gene is present. Flanking the cassette are two loxP sites which enable the removal of the Bsr resistance gene by the introduction of the protein Cre. (b) The gene replacement plasmid pLPBLP .

Figure 2: Strategy for construction of rnrA-in-pLPBLP

(a) The *rnrA* coding region and PCR products. The two flanking regions of *rnrA* were amplified using the primer pairs rnrA-5F-SalI/rnrA-5R-HindIII and rnrA-3F-PstI/rnrA-3R-BamHI. The fragments were called rnrA 5' and rnrA 3'. (b) rnrA 3' and pLPBLP. Both plasmid and insert was cut using the restriction endonucleases PstI and BamHI and the resulting plasmid was called rnrA3-in-pLPBLP. (c) rnrA 5' and nrA3-in-pLPBLP. The SalI and HindIII cut sites were used. (d) The gene replacement plasmid rnrA-in-pLPBLP.



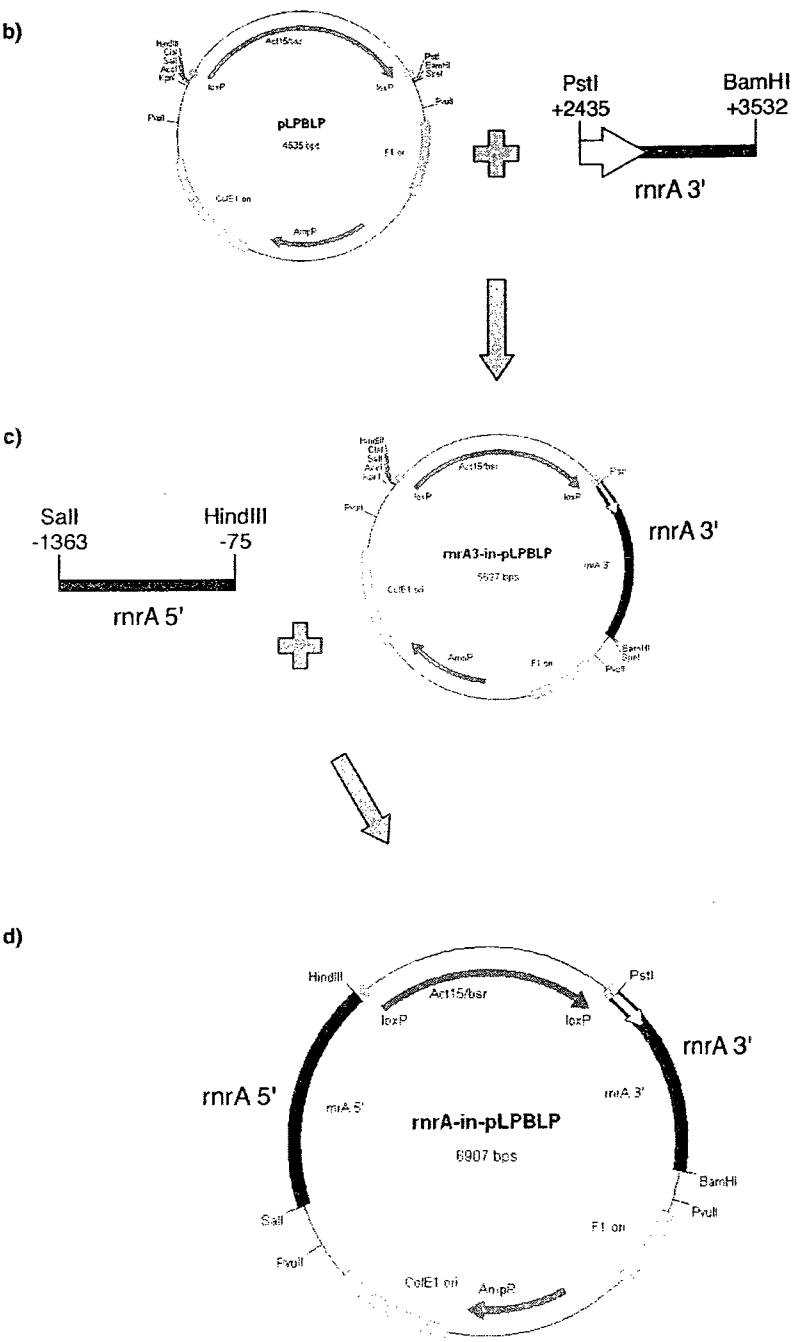
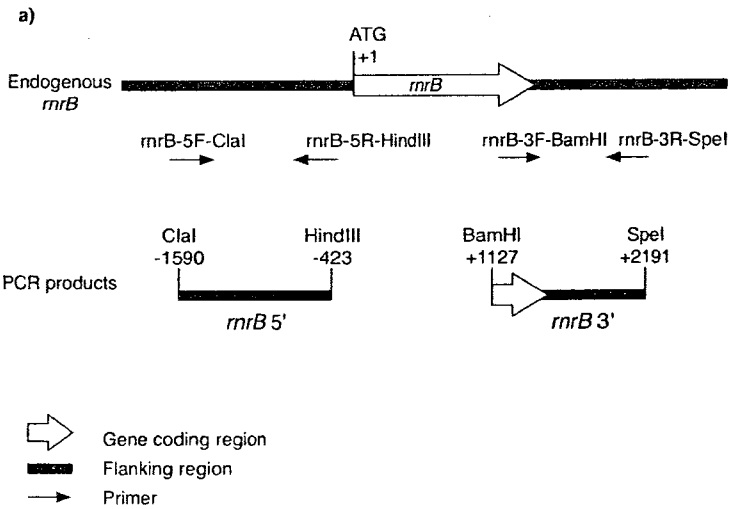
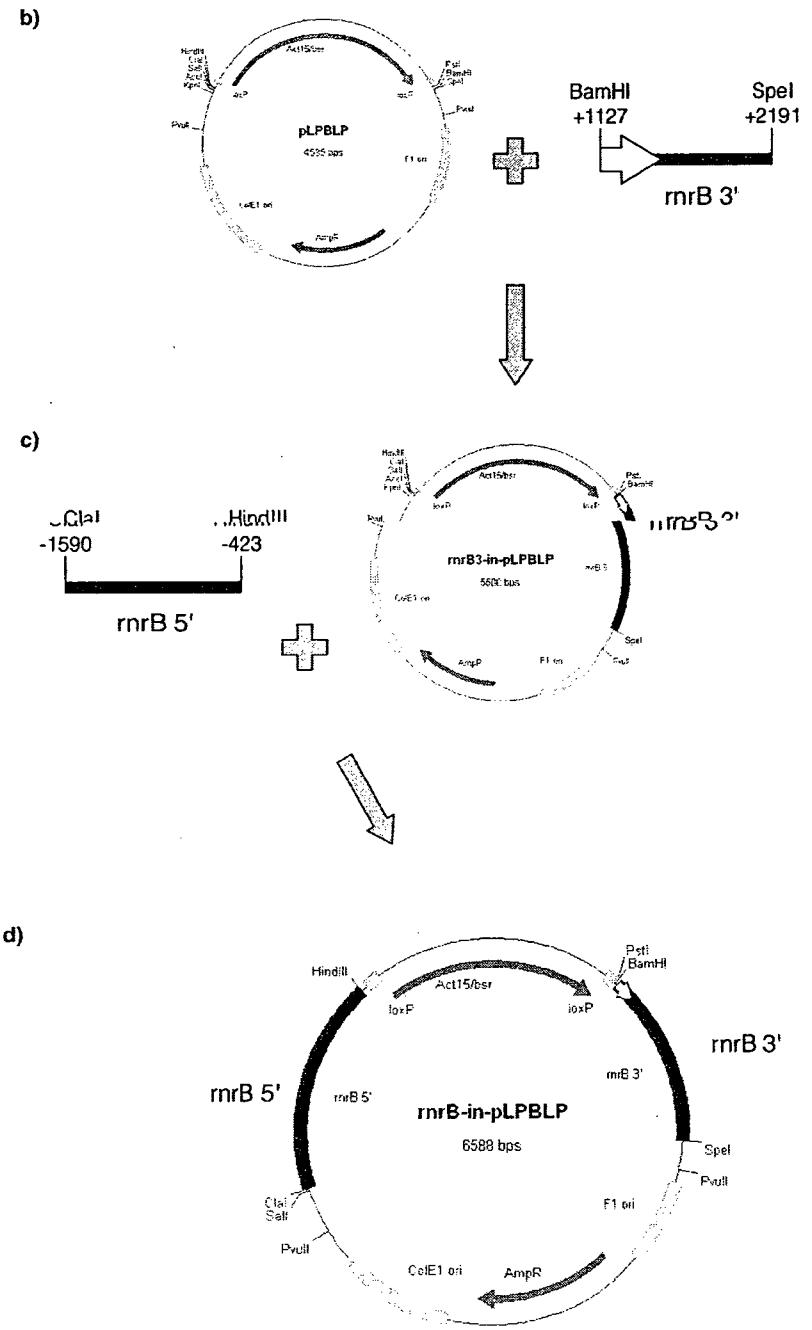

b)
pLPBLP
4535 bps
PstI
+2435
BamHI
+3532
rnrA 3'
c)
SalI
-1363
HindIII
-75
rnrA 5'
rnrA3-in-pLPBLP
5527 bps
rnrA 3'
d)
HindIII
Act15/bsr
loxP
loxP
PstI
rnrA 3'
rnrA 5'
rnrA 5'
rnrA 3'
rnrA-in-pLPBLP
6907 bps
BamHI
PvuII
F1 ori
SalI
PvuII
ColE1 ori
AmpR

Figure 3: Strategy for construction of rnrB-in-pLPBLP

(a) The endogenous rnrB and the PCR products rnrB 5' and rnrB 3'. The PCR products were amplified using the primer pairs rnrB-5F-ClaI/rnrB-5R-HindIII to generate the fragment rnrB5' and rnrB-3F-BamHI/rnrB-3R-SpeI to generate rnrB3'. (b) rnrB3' was cloned into pLPBLP. The restriction enzyme cut sites used to cut the plasmid and insert was BamHI and SpeI. (c) The plasmid rnrB3-in-pLPBLP. The plasmid was linearized with the restriction endonucleases ClaI and HindIII before rnrB 5', cut with the same restriction endonucleases, was cloned in. (d) The gene replacement plasmid rnrB-in-pLPBLP.




b)
pLPBLP
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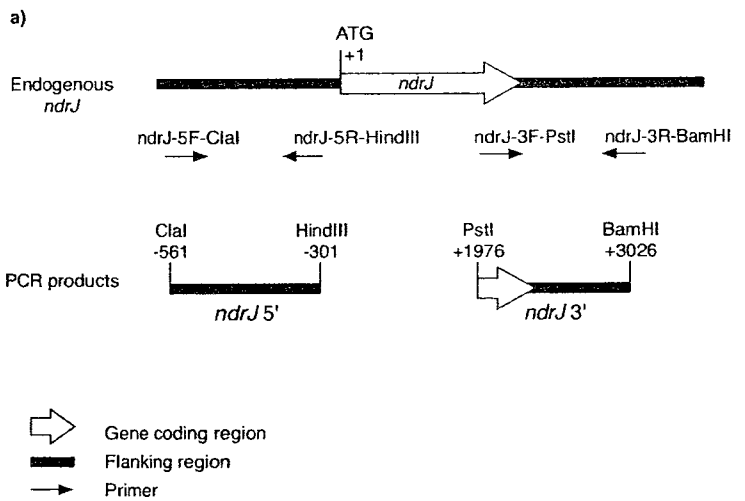
BamHI
+1127
SpeI
+2191
rnrB 3'
c)
ClaI
-1590
HindIII
-423
rnrB 5'
rnrB3-in-pLPBLP
5606 bps
d)
HindIII
PstI
BamHI
Act15/bsr
loxP
loxP
rnrB 3'
rnrB 5'
rnrB 5'
rnrB-in-pLPBLP
6588 bps
rnrB 3'
SpeI
PvuII
F1 ori
ClaI
SalI
PvuII
ColE1 ori
AmpR

Figure 4: Strategy for construction of ndrJ-in-pLPBLP

(a) The endogenous *ndrJ* and the PCR products ndrJ 5' and ndrJ 3'. ndrJ 5' was amplified using the primer pair ndrJ-5F-ClaI/ndrJ-5R-HindIII, while ndrJ-3F-PstI/ndrJ-3R-BamHI was used to amplify ndrJ 3'. (b) pLPBLP and ndrJ 3'. ndrJ 3' was cut and cloned into the plasmid pLPBLP using the PstI and BamHI restriction endonucleotide cut sites. (c) ndrJ-in-pLPBLP and ndrJ 5'. ndrJ 5' was cloned into the constructed plasmid using the ClaI and HindIII restriction endonucleotide cut sited. (d) The gene replacement plasmid ndrJ-in-pLPBLP



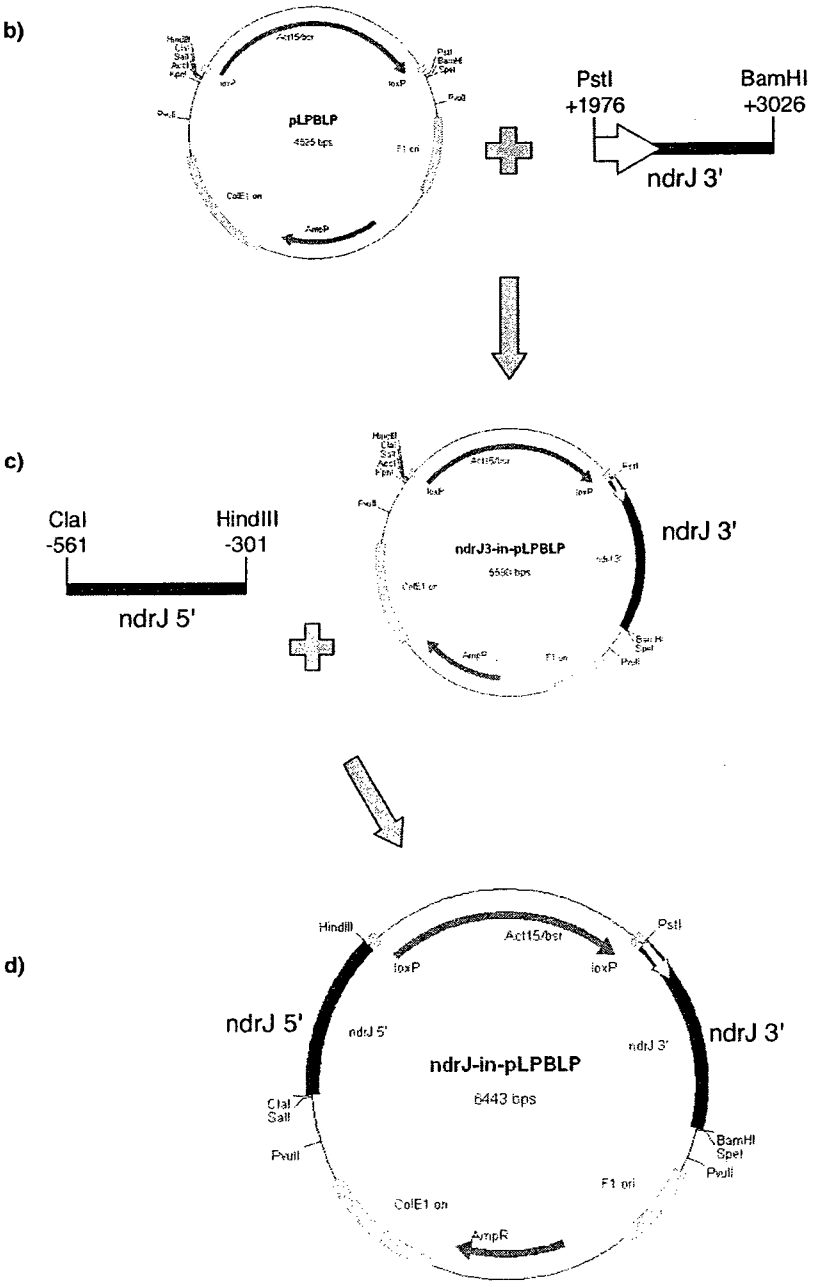
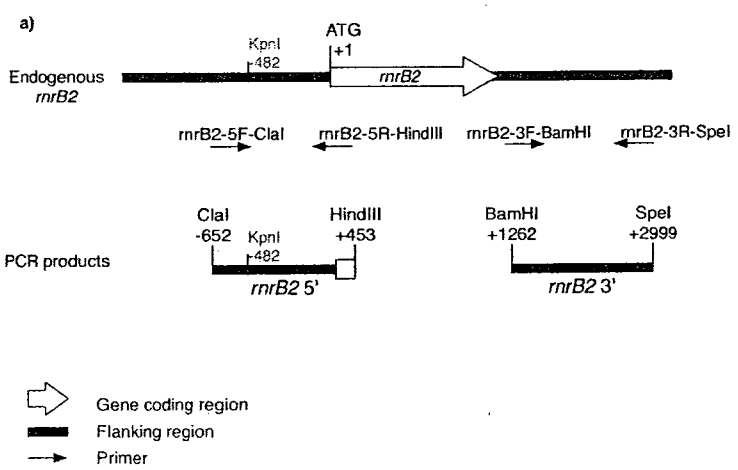
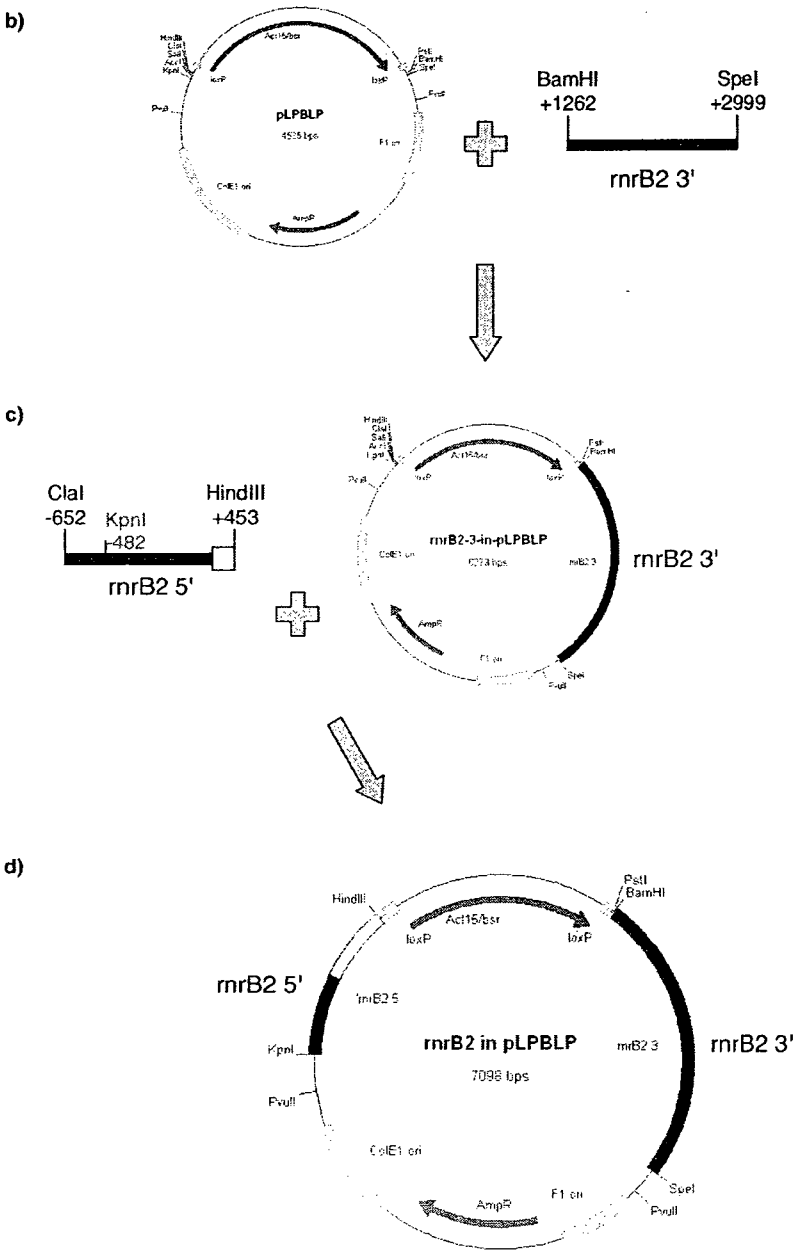

b)
pLPBLP
4525 bps
PstI
+1976
BamHI
+3026
ndrJ 3'
c)
ClaI
-561
HindIII
-301
ndrJ 5'
ndrJ3-in-pLPBLP
5563 bps
ndrJ 3'
d)
HindIII
Act15/bsr
PstI
loxP
loxP
ndrJ 5'
ndrJ 5'
ndrJ 3'
ndrJ 3'
ndrJ-in-pLPBLP
6443 bps
ClaI
SalI
PvuII
BamHI
SpeI
PvuII
F1 ori
ColE1 ori
AmpR

Figure 5: Strategy for construction of rnrB2-in-pLPBLP

(a) The endogenous *rnrB2* and the PCR products rnrB2 5' and rnrB2 3'. The 5' flanking region of rnrB2 was amplified using the primer pair rnrB2 5' was amplified using rnrB2-5F-ClaI7rnrB2-5R-HindIII to generate rnrB2 5, while the primers rnrB2-3F-BamHI/rnrB2-3R-SpeI was used to generate rnrB2 3'. (b) pLPBLP and rnrB2 3'. rnrB2 3' was cloned into the plasmid after being cut with the restriction endonucleases BamHI and SpeI. (c) rnrB2 3-in-pLPBLP and rnrB2 5'. The rnrB2 3' contained a ClaI cut site, and this restriction endonuclease could therefore not be used to linearize rnrB2 3-in-plpBLP. rnrB5' and rnrB 3'in'pLPBLP was therefore cut using the restriction endonucleases KpnI and HindII before ligated together. (c) The gene replacement plasmid rnrB2-in-pLPBLP.




b)
pLPBLP
BamHI
+1262
SpeI
+2999
rnrB2 3'
c)
ClaI
-652
KpnI
-482
HindIII
+453
rnrB2 5'
rnrB2-3-in-pLPBLP
rnrB2 3'
d)
HindIII
PstI
BamHI
Act15/bsr
loxP
loxP
rnrB2 5'
rnrB2 5
rnrB2 in pLPBLP
7098 bps
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rnrB2 3'
KpnI
PvuII
ColE1 ori
AmpR
F1 ori
SpeI
PvuII

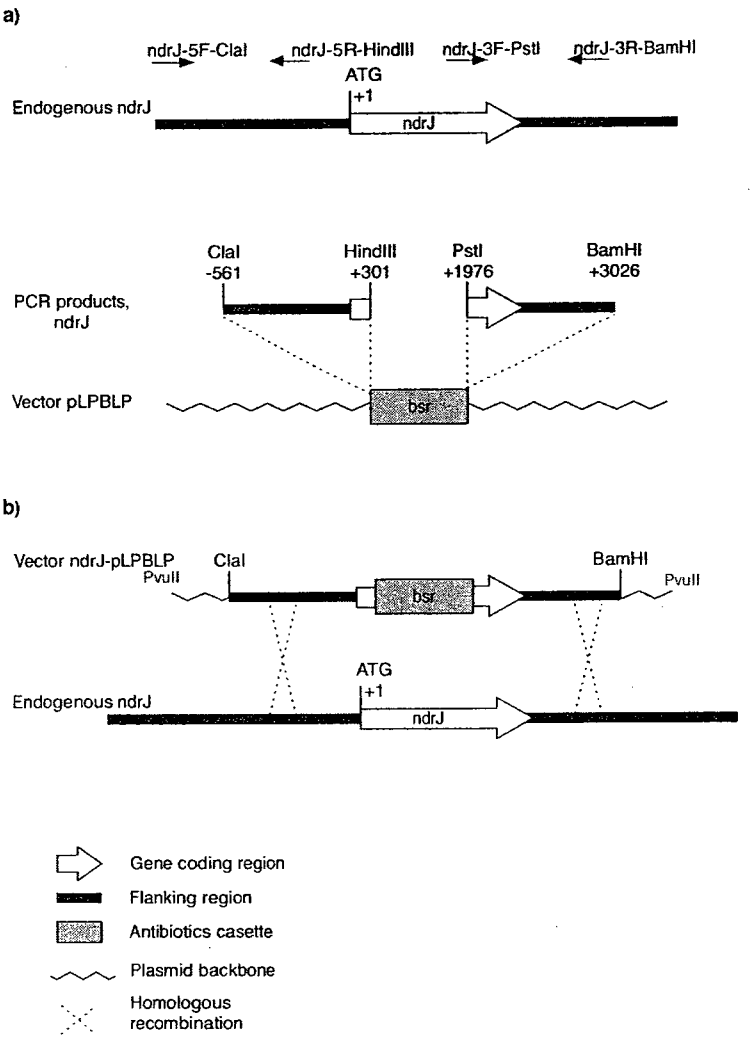


Figure 6: Gene replacement strategy of *ndrJ*.

(a) The flanking regions of *ndrJ* were PCR amplified and cloned into pLPBLP. (b) Homologous recombination of endogenous *ndrJ* and linearized plasmid. The Vector ndrJ-pLPBLP was linearized using the restriction endonuclease recognition sites flanking the Bsr cassette. The linearized plasmid was introduced into *Dictyostelium* cells. Homologous recombination between the linearized plasmid and the endogenous gene leads to a gene replacement where *ndrJ* is replaced with a truncated version and a Bsr cassette.

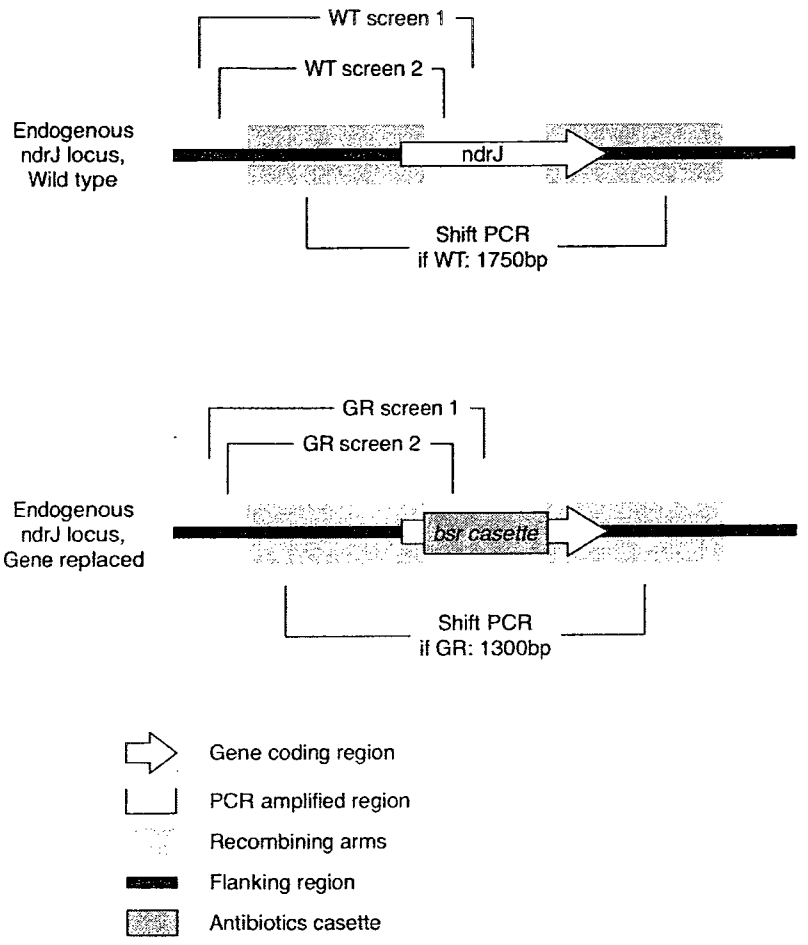


Figure 7: Binding sites for primers in PCR screen for gene replacement.

The primer pairs 1 and 2 were used in the initial screen for gene replacement. Two primer pairs were used for each strain. The WT pair amplifies wild type genes, while the GR pair would only give a product if the gene in question was replaced. The second round of PCRs were done with a primer pair that would bind within the recombining arms and amplify over the gene replaced region. A reduction in product size would be apparent if a gene replacement had taken place.

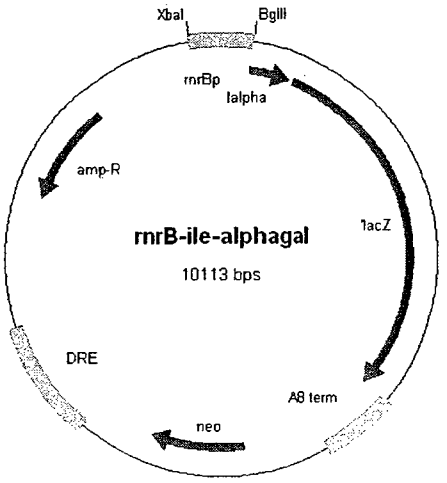


Figure 8: Physical map of rnrB-ile-αgal

The *rnrB*-ile-αgal plasmid has an unstable lacZ driven by the *rnrB* promoter. It also harbours a geneticin resistance gene and *Dictyostelium* Retransposable Elements that ensures chromosomal integration. The *rnrB* promoter is flanked by recognition sites for the restriction endonucleases XbaI and BglII that enables for excision of this promoter and insertion of another promoter of choice.

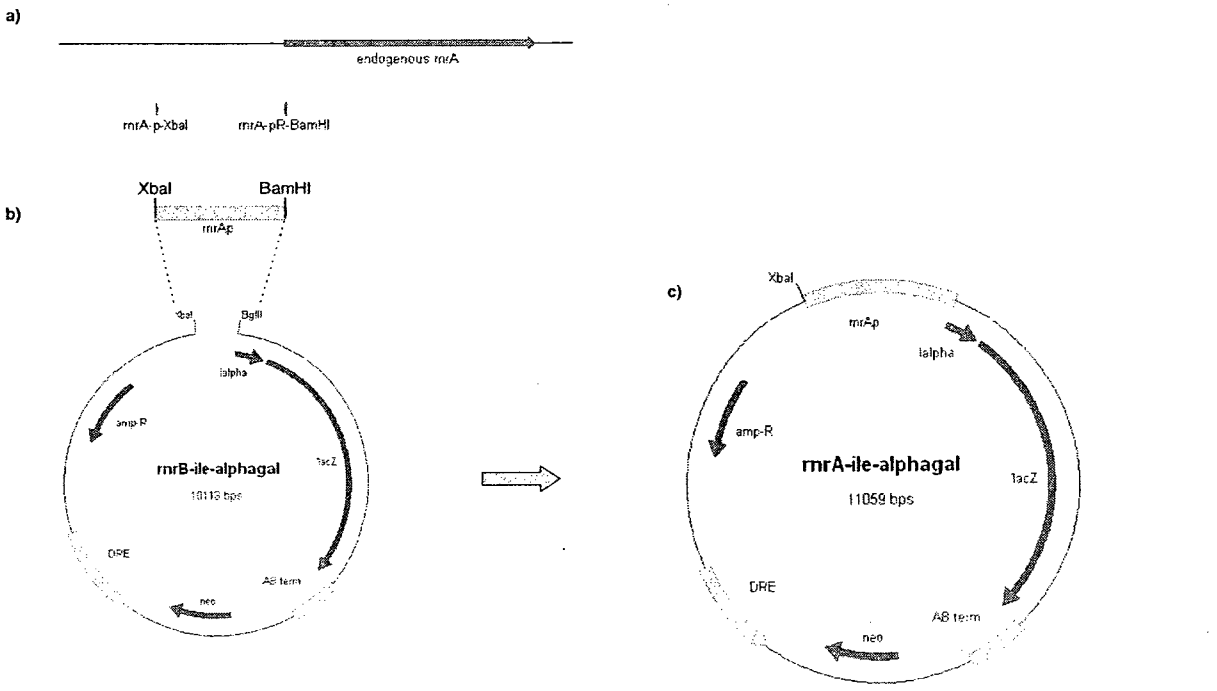


Figure 9: Cloning strategy for construction of rnrA-ile-αgal

(a) Endogenous *rnrA* and primer binding sites. The upstream region of the *rnrA* coding region was PCR amplified using the primer pair rnrA-p-XbaI and rnrA-pR-BamHI . (b) The PCR product rnrAp and rnrB-ile-αgal. The 1410 bp long PCR product rnrAp cut using XbaI and BamHI before cloned into rnrB-ile-αgal linearized with XbaI and BglII. (c) The expression vector rnrA-ile-αgal. This plasmid lacks the restriction endonuclease recognition site BglII found in the original plasmid rnrB-ile-αgal.

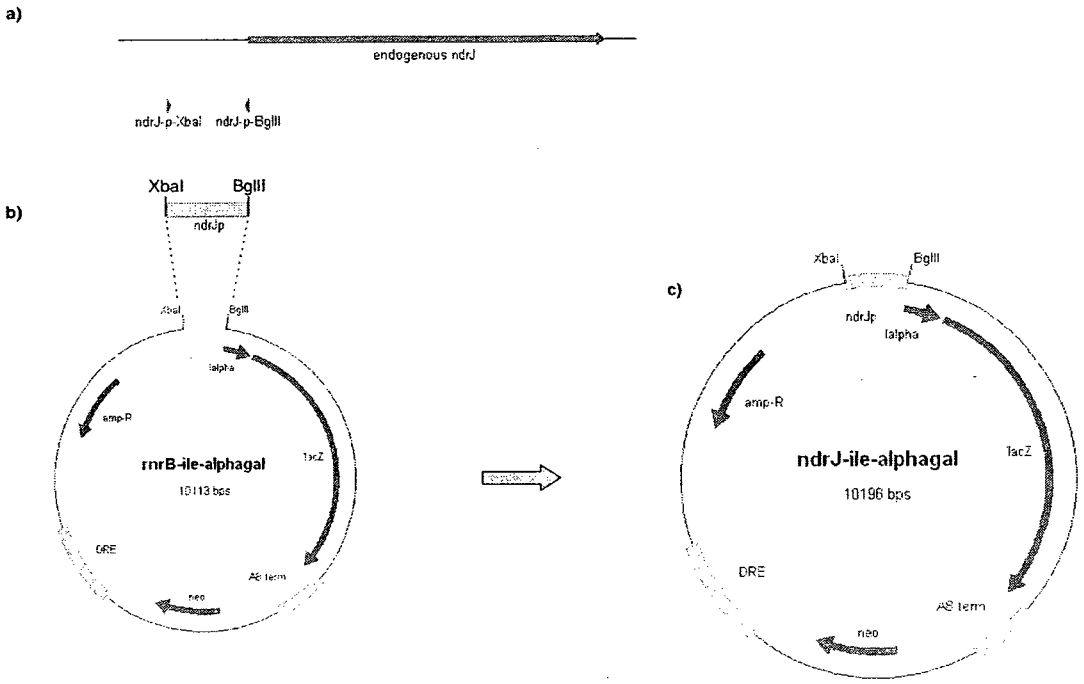


Figure 10: Cloning strategy for construction of ndrJ-ile-αgal

(a) Endogenpus *ndrJ* and primer binding sites. (b) rnrB-ile-αgal and ndrJp. The promoter region of *ndrJ* was amplified using the primer pair ndrJ-p-XbaI/ndrJ-p-BglII. The resulting fragment was 548 bp long and was cut with the restriction endonucleases XbaI and BglII before cloned into rnrB-ile-αgal linearized with the same enzymes. (c) The expression vector ndrJ-ile-αgal.

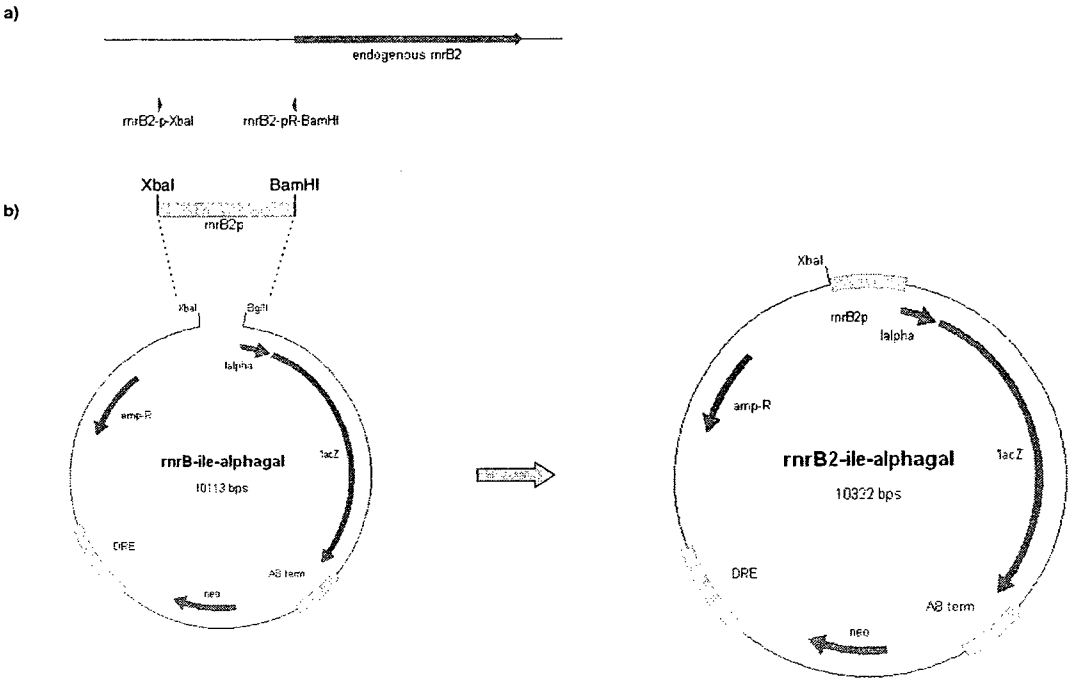


Figure 11: Cloning strategy for construction of rnrB2-ile-αgal

(a) Endogenous *rnrB2* and primer binding sites. (b) The PCR product rnrB2p and plasmid rnrB-ile-αgal. rnrB2p was amplified from the 5' region of the rnrB2 coding region using PCR and the primer pair rnrB2-p-XbaI and rnrB2-pR-BamHI. The resulting fragment was 676 bp long and was cut with the restriction endonucleases XbaI and BamHI. The cut fragment was cloned into rnrB-ile-αgal linearized uding XbaI and BglII. (c) The expression vector rnrB2-ile-αgal. This plasmid lacks the endonuclease recognition site BglII found in the original plasmid rnrB-ile-αgal.

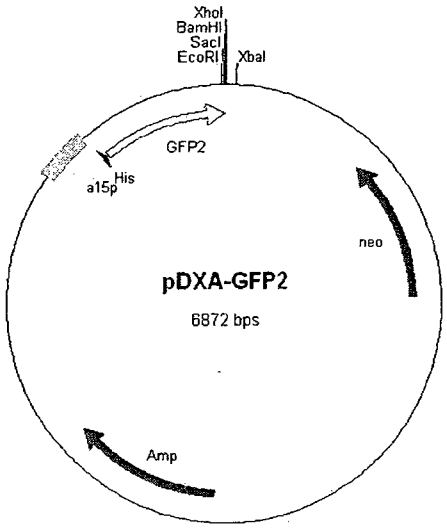


Figure 12: Physical map pDXA-GFP2

pDXA-GFP2 contains a GFP2 driven by the Actin 15 promoter. The translated protein will have a His tag on its C terminus. A multiple cloning site downstream of the GFP2 coding sequenced enables for cloning of genes in frame with the fluorescent protein where the produced GFP2 will have the protein of interest fused to the N-terminus. The plasmid also has resistance genes for ampicillin and geneticin (neomycin).

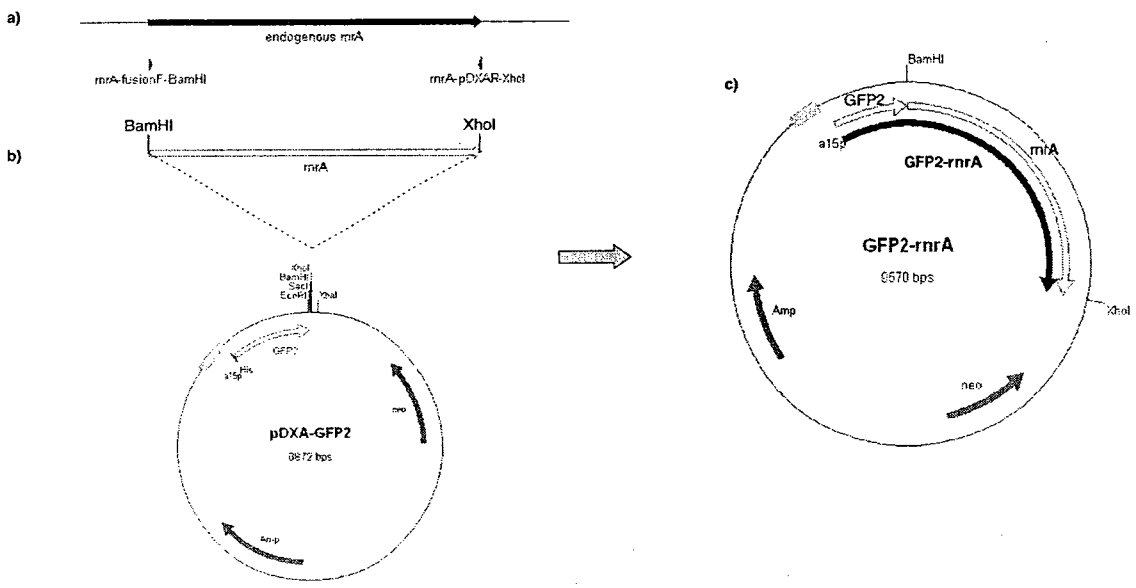


Figure 13: Strategy for construction of GFP2-rnrA

(a) Endogenous *rnrA* and primer binding sites. The primer pair rnrA-fusionF-BamHI/rnrA-pDXAR-XhoI was used to PCR amplify the full length *rnrA* coding region. (b) Amplified rnrA and pDXA-GFP2. The PCR product was cut using BamHI and XhoI and cloned into the corresponding recognition sites in pDXA-GFP2. (c) The expression vector GFP2-rnrA. When translated, the GFP2 will have RnrA fused to its C terminus.

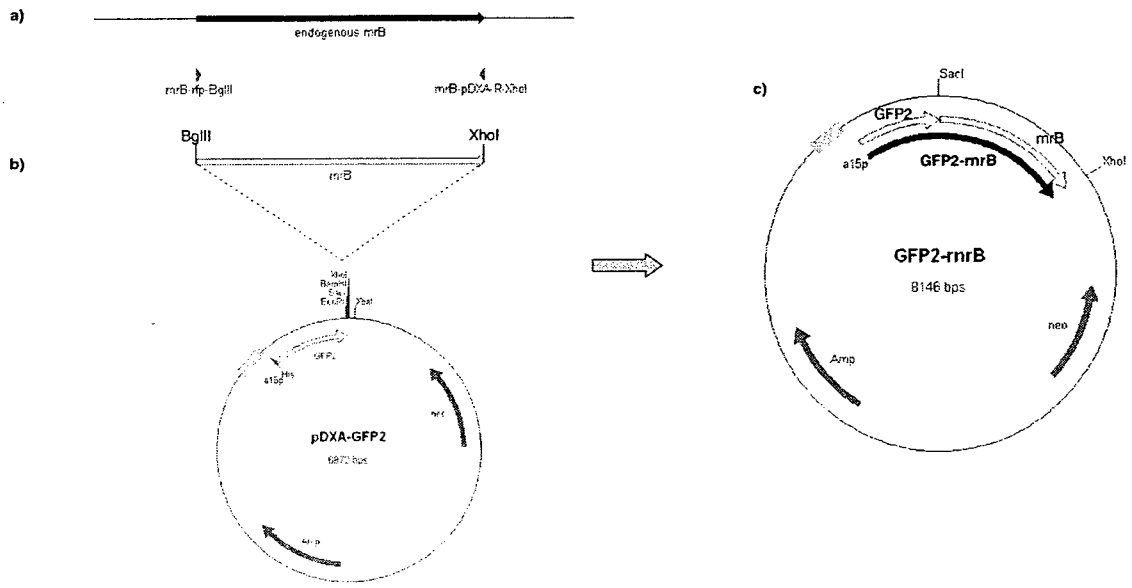


Figure 14: Strategy for construction of GFP2-rnrB

(a) Endogenous *rnrB* and primer binding sites. Full length *rnrB* was amplified using PCR and the primer pair rnrB-rfp-BglII/rnrB-pDXA-R-XhoI. (b) Amplified *rnrB* and pDXA-GFP2. The PCR product was cut using the restriction endonucleases BglII and XhoI, while BamHI and XhoI was used to linearize the plasmid before they were ligated together. (c) The expression vector GFP2-rnrB. The resulting vector will encode a GFP with RnrB fused to its C-terminus. The BamHI recognition site present in the original plasmid will be removed in the cloning process.

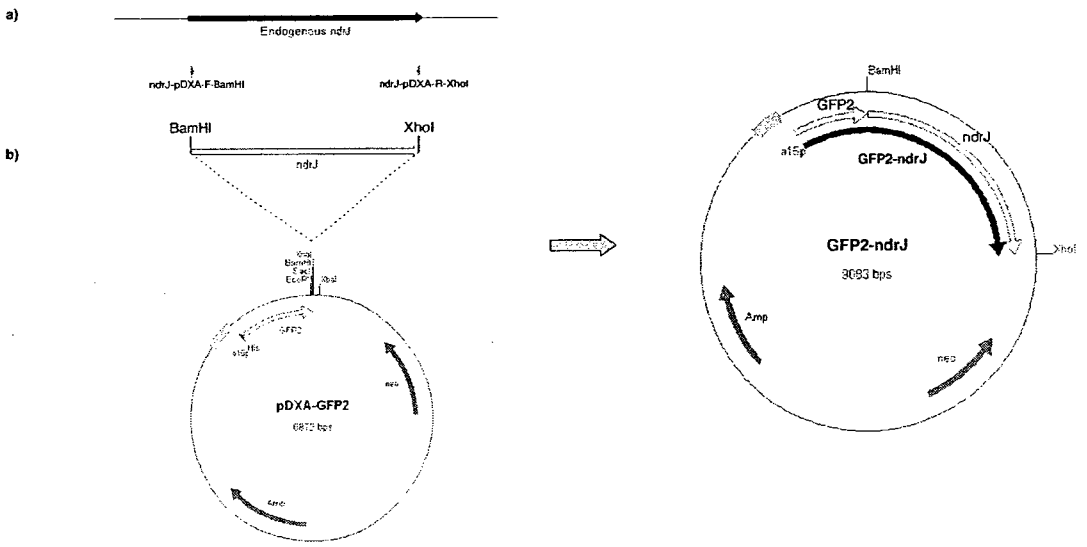


Figure 15: Strategy for construction of GFP2-ndrJ

(a) Endogenous *ndrJ* and primer binding sites. (b) Amplified *ndrJ* and pDXA-GFP2. The fill length *ndrJ* was amplified using PCR and the primer pair ndrJ-pDXA-F-BamHI/ndrJ-pDXA-R-XhoI before cut with the restriction endonucleases BamHI and XhoI and cloned into the corresponding sites in the plasmid pDXA-GFP2. (c) The expression vector GFP2-ndrJ. When the GFP is transcribed it will have NdrJ fused to its C-terminus.

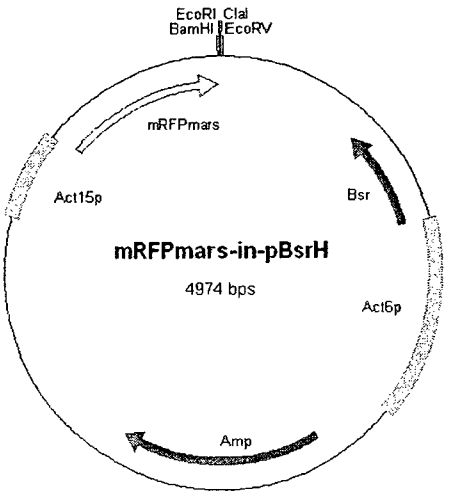


Figure 16: Physical map of mRFPmars-in-pBsrH

mRFPmars-in-pBsrH contains a red fluorescent protein, mRFPmars driven by the Actin 15 promoter. Downstream of the mRFPmars coding region is a multiple cloning site, which enables for insertion of genes in frame with mRFPmars. The plasmid also has resistance genes to the antibiotics ampicillin and blasticidin.

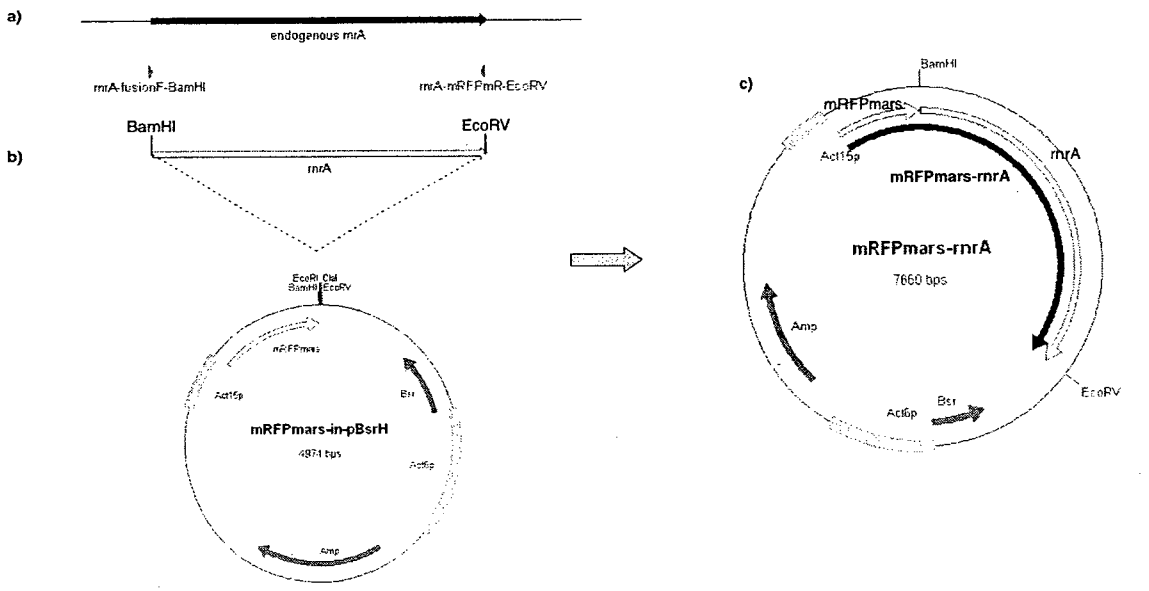


Figure 17: Strategy for construction of mRFPmars-rnrA

(a) Endogenous *rnrA* and primer binding sites. (b) Amplified *rnrA* and mRFPmars-in-pBsrH. The full length *rnrA* was amplified using PCR and the primer pair rnrA-fusionF-BamHI/*rnrA*-mRFPmarsR-EcoRV. The forward primer is the same as used in the construction of GFP2-rnrA. The PCR product was cut and cloned into the mRFP-mars-in-pBsrH with the aid of restriction enzyme digest using BamHI and EcorV. (c) The expression vector mRFPmars-rnrA. The vector encodes an RFP with RnrA fused to its C-terminus.

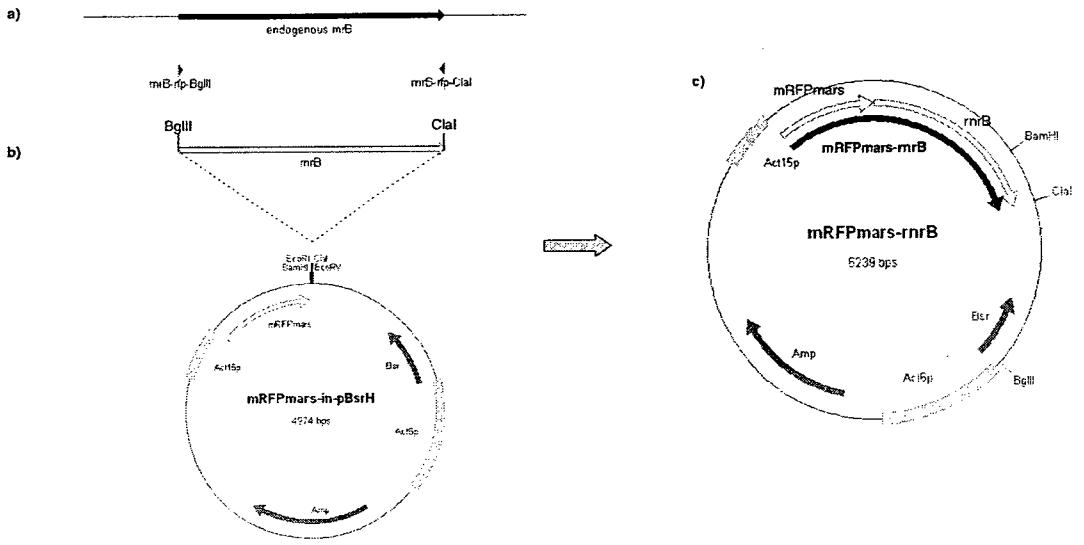


Figure 18: Strategy for construction of mRFPmars-rnrB

(a) Endogenous *rnrB* and primer binding sites. (b) Amplified *rnrB* and mRFPmars-in-pBsrH. The full length *rnrB* was amplified using the primer pair rnrB-rfp-BglII/rnrB-rfp-ClaI. The forward primer is the same as used in the construction of GFP2-rnrB. The PCR product was cut using the restriction endonucleotides BglII and ClaI and cloned into mRFPmars-in-pBsrH linearized with the enzymes BamHI and ClaI. (c) The expression vector mRFPmars-rnrB. The vector encodes an RFP with RnrB fused to its C-terminus. The BamHI recognition site present in the original plasmid has been removed.

Table 1: Primers designed for use in PCRs to generate fragments for construction of plasmids for homologous recombination.

	Primer	Sequence	Used to construct plasmid
1	rnrA-5F-SalI	ACGC**GTCGAC**TCTGCTGCATTACTGTCC	
2	rnrA-5R-HindIII	CGC**AAGCTT**GGAGTGTTGGTTGTAACC	
3	rnrA-3F-PstI	AA**CTGCAG**CCAATCGTATCTCAAACTCC	
4	rnrA-3R-BamHI	CG**GGATCC**CACCTATTCTATGTTGAGG	rnrA-pLPBLP
5	rnrB-5F-ClaI	CC**ATCGAT**ATAACCTCCTGGTGCTCC	
6	rnrB-5R-HindIII	CCC**AAGCTT**CTATATTTGGGTACCACTCC	
7	rnrB-3F-BamHI	CG**GGATCC**CTGGTGTTGCAATTCAAGG	
8	rnrB-3R-SpeI	G**ACTAGT**CACCAATTTCAGCAGAACC	rnrB-pLPBLP
9	ndrJ-5F-ClaI	CC**ATCGAT**GAAGAGATCTACCAACAACC	
10	ndrJ-5R-HindIII	CCC**AAGCTT**CACCCAATACTCCAAATCC	
11	ndrJ-3F-PstI	AT**CTGCAG**GTACAGTCTCTTTTGATCC	
12	ndrJ-3R-BamHI	CG**GGATCC**TCAATCACGAACCAGACG	ndrJ-pLPBLP
13	rnrB2-5F-ClaI	CC**ATCGAT**TGTTCCTTGGAAGTCACC	
14	rnrB2-5R-HindIII	CCC**AAGCTT**GGAGAGTTGATATGATTGG	
15	rnrB2-3F-BamHI	CG**GGATCC**AACAATCATAGGCTACGC	
16	rnrB2-3R-SpeI	G**ACTAGT**CATATTAAAGGTGTTTCTGG	rnrB2-t-pLPBLP

Note: overhangs are underlined while restriction endonuclease recognition sites are in bold.

Table 2: Primers designed for use in PCRs to generate fragments for construction of plasmids for *in situ* and localization experiments.

	Primer	Sequence	used to construct plasmid
17	rnrA-p-XbaI	ATCTAGAATCTGCTGCATTACTGTCC	
18	rnrA-pR-BamHI	CGGGATCCTGGAGTTACATTAATACTATTAC	rnrAp-ile-agal
19	ndrJ-p-XbaI	ATCTAGACTCTCAGTATCAATCTTGG	
20	ndrJ-p-BglII	TGAAGATCTCATATTTTGTTGTTATCTC	ndrJp-ile-agal
21	rnrB2-p-XbaI	CTCTAGAATTGTTCCTTGGAAGTCACC	
22	rnrB2-pR-BamHI	CGGGATCCTATCATTTTTTGGGGAATATG	rnrB2p-ile-agal
23	rnrA-fusionF-BamHI	CGCGGATCCAGTAATAGTATTAATGTAACTCC	GFP2-rnrA, mRFPmars-rnrA
24	rnrA-pDXAR-XhoI	CCGCTCGAGATTAACTACCACAAACTAAAC	GFP2-rnrA
25	rnrA-mRFPmR-EcoRV	AGATATCTTAACTACCACAAACTAAAC	mRFPmars-rnrA
26	rnrB-rfp-BglII	GAAGATCTCATACATTCATTGAACCAATTC	GFP2-rnrB, mRFPmars-rnrB
27	rnrB-pDXA-R-XhoI	CCGCTCGAGGCTTCATCTAAAACTAAAGTTC	GFP2-rnrB
28	rnrB-rfp-ClaI	CCATCGATCATCTAAAACTAAAGTTCTTG	mRFPmars-rnrB
29	ndrJ-pDXA-F-BamHI	ATGGATCCCTATTTAAAAGTAGTTGTAATTCC	
30	ndrJ-pDXA-R-XhoI	CATCTCGAGCAGAAACAATTGTACAACTTG	GFP2-ndrJ

Note: overhangs are underlined while restriction endonuclease recognition sites are in bold.

Table 3: Settings of Thermal Cycler for PCR amplification of gene fragments according to primer pair.

Primers		Fragment length (bp)	Step1: Annealing		Step2: Annealing		Extension		Fragment used in plasmid
Forward primer	Reverse primer		Temp (°C)	Number of cycles	Temp (°C)	number of cycles	temp (°C)	time (min)	
rnrA-5F-SalI	rnrA-5R-HindIII	1289	49	10	57	20	66	2.3	
rnrA-3F-PstI	rnrA-3R-BamHI	1098	45	10	50	20	68	2.3	rnrA-pLPBLP
rnrB-5F-ClaI	rnrB-5R-HindIII	1168	55	10	57.5	20	68	2.3	
rnrB-3F-BamHI	rnrB-3R-SpeI	965	45	10	50	20	68	2.3	rnrB-pLPBLP
ndrJ-5F-ClaI	ndrJ-5R-HindIII	863	45	10	50	20	68	2.3	
ndrJ-3F-PstI	ndrJ-3R-BamHI	1051	40	10	45	20	63	2.3	ndrJ-pLPBLP
rnrB2-5F-ClaI	rnrB2-5R-HindIII	1106*	45	10	50	20	68	2.3	
rnrB2-3F-BamHI	rnrB2-3R-SpeI	1738	45	10	52	20	63	2.3	rnrB2-pLPBLP
rnrA-p-XbaI	rnrA-pR-BamHI	1410	45	10	55	20	63	2.3	rnrA-ile-agaI
ndrJ-p-XbaI	ndrJ-p-BglII	548	43	10	54	20	68	2.3	ndrJ-ile-agaI
rnrB2-p-XbaI	rnrB2-pR-BamHI	676	44	10	55	20	68	2.3	rnrB2-ile-agaI
rnrA-fusionF-BamHI	rnrA-pDXAR-XhoI	2717	47	7	61	18	63	4	GFP2-rnrA
rnrB-rfp-BglII	rnrB-pDXA-R-XhoI	1292	47	7	61	18	63	2	GFP2-rnrB
ndrJ-pDXA-F-BamHI	ndrJ-pDXA-R-XhoI	2229	40	12	50	20	63	2.6	GFP2-ndrJ
rnrA-fusionF-BamHI	rnrA-mRFPmR-EcoRV	2714	47	7	61	18	63	4	mRFPmars-rnrA
rnrB-rfp-BglII	rnrB-rfp-ClaI	1287	40	10	50	20	63	2.6	mRFPmars-rnrB

*An internal KpnI site was used in cloning. Cut fragment is 935bp.

3 Results

3.1 Generation of gene replacement strains

3.1.1 Gene disruption of *ndrJ*

To investigate the physiological function of *ndrJ*, the gene was replaced with an antibiotics cassette as illustrated in Figure 6. Screening for gene replacement strains was carried out using PCR amplification of the target gene region. Several different primer pairs were used (see Figure 7 for representation of primer binding sites relative to the gene). Primer pair number 3 bound to the chromosome in such a way that the product would shift in size from 1750 bp in wild type cells to 1300 bp in a gene replaced strain. Three gene replacement strains were obtained and named Δ*ndrJ*::bsR no.1-3 (see Figure 19). Two independent transformants with intact *ndrJ,* but with the antibiotics cassette present, were put aside as random integration controls. These were called AX2-bsR.

To monitor if removal of *ndrJ* had any effect on the cell cycle, increase in cell number over the course of two to three normal generations were monitored. Exponentially growing cells from cultures of Δ*ndrJ*::bsR no.1 and 2 were counted using a haemocytometer and compared to AX2 and a random integration control, AX2-bsR. The two controls showed a similar growth pattern to each other and had a generation time of 10 to 11 hours. The two gene replacement strains examined behaved very differently. While Δ*ndrJ*::bsR no.1 displayed close to normal generation time of 12 hours,

Δ*ndrJ*::bsR no.2 showed a retarded growth pattern with an estimated generation time of 26 hours (see Figure 20). This pattern was reproduced over three experiments.

3.1.2 No *rnrB* gene replacement strain was obtained

Only a few antibiotics-resistant cells were recovered from each transformation using the rnrB gene replacement construct, independent of minor changes to the protocol such as increasing DNA concentration. Twenty independent transformants were screened, but the desired gene replacement was not obtained. The *rnrB* gene and its flanking region have repeatedly shown itself problematic to amplify using PCR and this difficulty was more pronounced in transformed cells. Together, these obstacles impeded further attempts to disrupt the gene.

3.1.3 Gene disruption of *rnrB2*

Following electroporation with linearized rnrB2-pLPBLP, 5 antibiotic resistant cell lines were screened using PCR. The primers used bound to the gene in the same manner as the primers used to screen for gene replaced *ndrJ*. One strain positive for the gene replacement was obtained and named *ΔrnrB2*::bsR.

3.2 Spatial expression of RNRs in developing structures

The *rnrB* promoter is the only RNR promoter that has been experimentally analyzed in *Dictyostelium* and four *cis*-regulating elements have been localized (Bonfils, 1999). This promoter does not look like any other known promoters and which factors actually bind to these sites are unknown (Bonfils, 1999). Expression of this gene has also been

localized to the prespore cells (Tsang, 1996), but it is unknown where in the migrating slugs the other RNRs are expressed.

To examine where in the developing structures the promoters are active, the reporter gene encoding β-galactosidase was set under the control of the different *rnr* promoters. Cleavage of X-gal by β-galactosidase yields a blue colour and this assay can therefore be used as a visual indicator for where in the structure promoter activity is found. This also gives an opportunity to examine if the spatial pattern of gene expression differs between different *rnr* promoters and between different *Distyostelium* strains.

Figure Figure 21a shows developing AX2 cells without any inserted plasmid, while Figure 21b shows that cells carrying the rnrA-ile-αgal plasmid produced β-galactosidase in the prespore regions only. The expression pattern of *rnrA* was altered in the *rblA*-null strain as β-galactosidase controlled by the *rnrA* promoter was expressed in the entire multicellular structures (Figure 21c).

rnrB has previously been studied in AX2 and it was determined that the expression is prespore specific (Tsang, 1996), also see Figure 21d. Even though *rnrB* expression is higher in the *rblA*-null strain (K. Straβer, personal communication), the spatial expression pattern remains the same; production of β-galactosidase driven by the *rnrB* promoter is localized to the prespore regions of the structures.

Cells transformed with the plasmids ndrJ-ile-αgal and rnrB2-ile-αgal were starved and developed as above, but no β-galactosidase production was detected (see Figure 21f and Figure 21g for images of cells carrying the ndrJ-ile-αgal construct. *rnrB2*/lacZ producing cells are not shown).

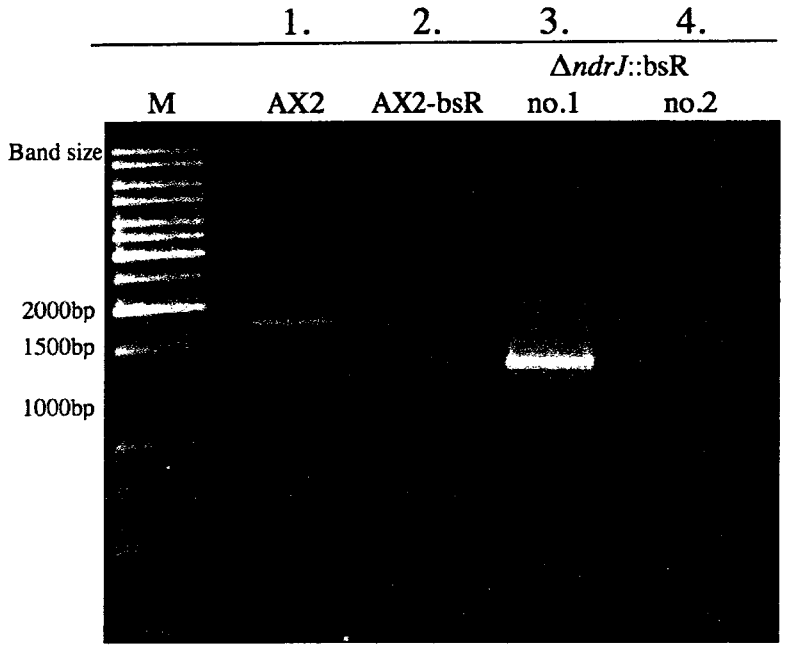


Figure 19: PCR analysis of genomic DNA from wild type cells versus gene replaced *ndrJ*

The primer pair used bound to the genomic DNA in the region flanking the replaced region. A PCR product would be of different size depending on whether the strain is wild type or the gene has been replaced: the PCR product of the wild-type allele of *ndrJ* is expected to be 1750 bp while the replaced gene construct is 1300 bp. The primers would also bind to the gene replacement plasmid, ndrJ-in-pLPBLP and a PCR product would be 1300 bp. In Lane 1, wild-type AX2 cells; lane 2, a transformant with two PCR products suggesting the replaced gene construct was randomly integrated; lanes 3 and 4, gene replacement strains producing only the 1300-bp PCR product.

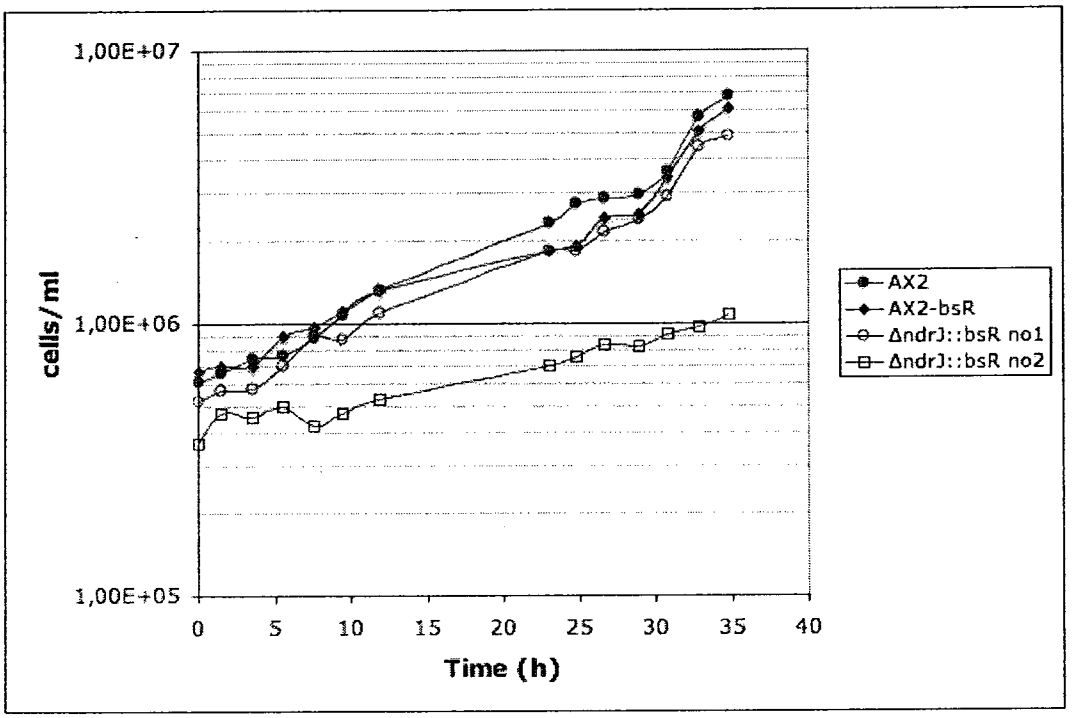


Figure 20: Growth curve to estimate cell cycle length of Δ*ndrJ*::bsR.

Exponentially growing cells of AX2, AX2-bsR and Δ*ndrJ*::bsR no.1 and no.2 strain were counted using a haemocytometer over a course of 35 hours to get an estimate of cell cycle length. Calculated cell cycle length is 10 hours for AX2, 11 hours for AX2-bsR, 12 hours for Δ*ndrJ*::bsR no.1 and and 25 hours for *ndrJ*::bsR no.2.

The doubling times were calculated using the formula ($r = (\log(N_1/N_0))/t$) where N is cell number at a given time t is the number of hours between cell counts. Three growth experiments were done and the doubling times given are averages from multiple calculations from each experiment.

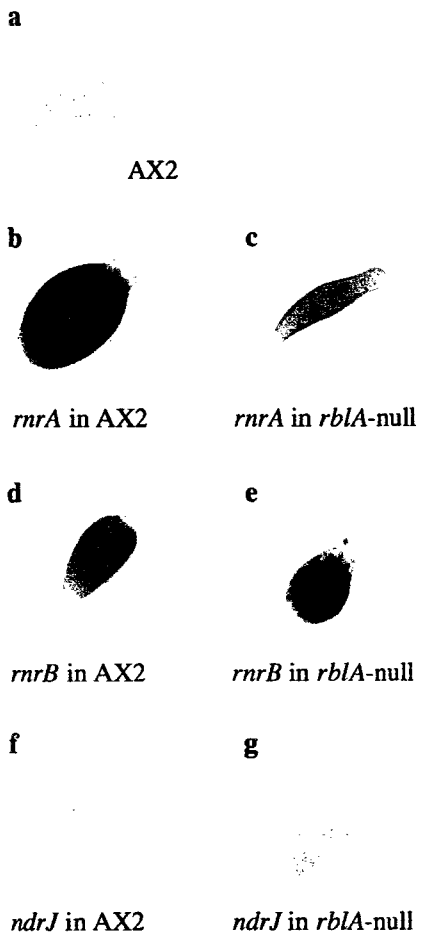


Figure 21: Histological staining of AX2 and *rblA*-null cells producing β-galactosidase

Cells producing β-galactosidase controlled by *rnrA*, *rnrB* and *ndrJ* promoters were starved for 16-18 hours before being fixed and assayed for β-galactosidase activity. Panel a shows wild type cells, AX; panel B shows rnrA expression in AX2; panel c shows rnrA expression in *rblA*-null cells; panel d shws *rnrB* expression in AX2; panel e shows *rnrB* expression in *rblA*-null cells; panel f shows *ndrJ* expression in AX2; panel g shows *ndrJ* expression in *rblA*-null cells. Note that the anterior region, containing prestalk cells is pointing up towards the right.

3.3 Imaging of fluorescent fusion proteins

3.3.1 Effects of sample preparation

Dictyostelium cells are usually imaged as tissue cultures: live using an inverted microscope (Müller-Taubenberger, 2006). The cells can be mounted under a thin layer of agarose to keep them flat and the air space between the cover slip and the microscope slide can supply the cells with enough air to survive for a week (Fukui, 1987). Since both the microscopes available for these experiments were epifluorescence microscopes, other means had to be used to image healthy cells, either alive or fixed.

The axenic medium used to grow *Dictyostelium* cells was fluorescent in itself, mainly in the emission spectra of GFP (Engel, 2006). The medium was retained in vacuoles within the cells and the auto-fluorescence could not be reduced by washing the cells. To give the cells an opportunity to degrade the auto-fluorescent vacuoles, they were grown in Loflo+yeast medium for 1-2 hours prior to imaging (Fey, 2009). These cells could then be observed live without interfering background signal. Unfortunately, growing cells have a very limited life span when sandwiched between a microscope slide and a cover slide; the cells started dying approximately 15 minutes after mounting, probably due to lack of oxygen.

To get more time for observation with each prepared microscope slide, different fixation techniques were attempted. Methanol and methanol with 1% or 4% formaldehyde was initially tried, but both these fixation techniques lead to a complete loss of fluorescent signal.

The most efficient way of fixing cells was found to be ethanol fixation at room temperature. The fluorescent signal was weaker in these cells compared to live cells, but it allowed for some storage time and opportunity to observe the cells for longer than 15 minutes. Growing cells and cells in early development were successfully imaged in this manner (see Figure 22 and Figure 24).

3.3.2 The RNRs are sequestered to different parts of the cell

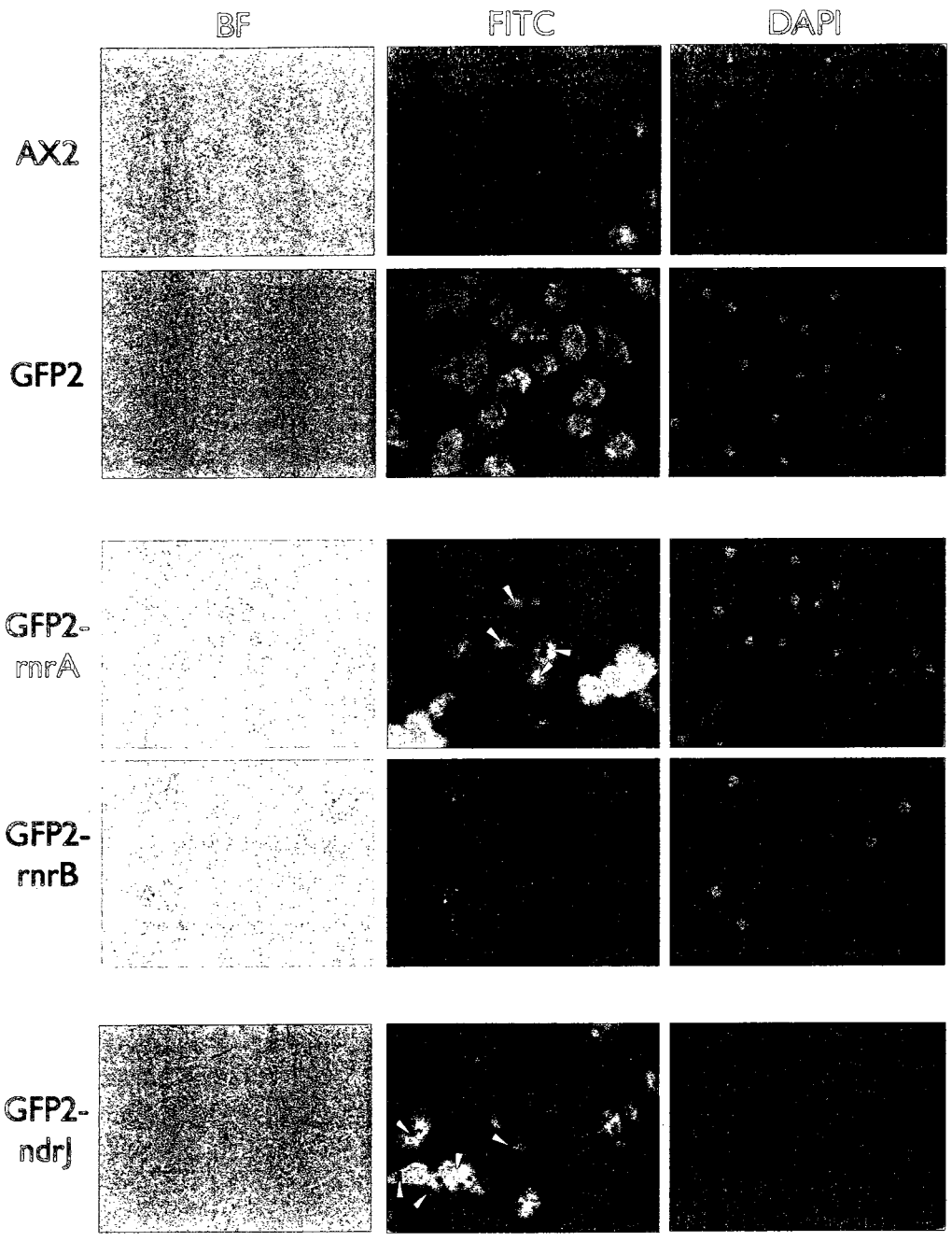
Exponentially growing cells were incubated in Loflo+yeast for 1 to 2 hours before being fixed with ethanol. The DNA was stained with DAPI and the cells mounted for imaging. Each of the strains carrying an expression plasmid was compared to two controls, one wild type strain and one wild type strain transformed with the empty plasmid pDXA-GFP2. The controls were exposed for the same amount of time as the strain being studied. Figure 22 shows that RnrA was mainly localized to the nucleus, while RnrB was found in a granular pattern in the cytoplasm. The number of granules differed between cells, from one bright granule to several smaller granules. None of these granules were localized to the mitochondria or the nucleus (see Figure 23). In contrast to the GFP2-rnrA fusion protein, the NdrJ enzyme was found in the entire cytoplasm of the cells with a noticeable exclusion from the nucleus and some of the vacuoles (see Figure 22).

To examine whether the localization pattern would change in early development, at a point where the RNRs are usually not expressed, the cells were starved for 4 to 5 hours, fixed and observed as above (see Figure 24). GFP2-rnrA and GFP2-ndrJ remained localized in the same manner as growing cells, except for a reduction in signal strength. GFP2-rnrB, however, changed localisation pattern in early development compared to

growing cells: the few, bright granules present in growing cells were replaced with a higher number of smaller granules (see Figure 25 for a larger image of the GFP2-rnrB).

Figure 22: Localisation of RNRs in growing cells

The cells were incubated in Loflo+yeast before being fixed with ethanol. The AX2 is a wild type strain, while the GFP2 strain is a wild type strain transformed with the empty vector. The GFP2 only is found in the cytoplasm and some of the nuclei, but is excluded from the vacuoles in the cell. GFP2-rnrA localizes to the nucleus of the cells, while the GFP2-rnrB displays a granular pattern in the cytoplasm. GFP2-ndrJ is excluded from the nucleus and found in the cytoplasm. All images are taken with a 100x objective. White arrows point at the nuclei of the cells.


BF
FITC
DAPI
AX2
GFP2
GFP2-
rnrA
GFP2-
rnrB
GFP2-
ndrJ

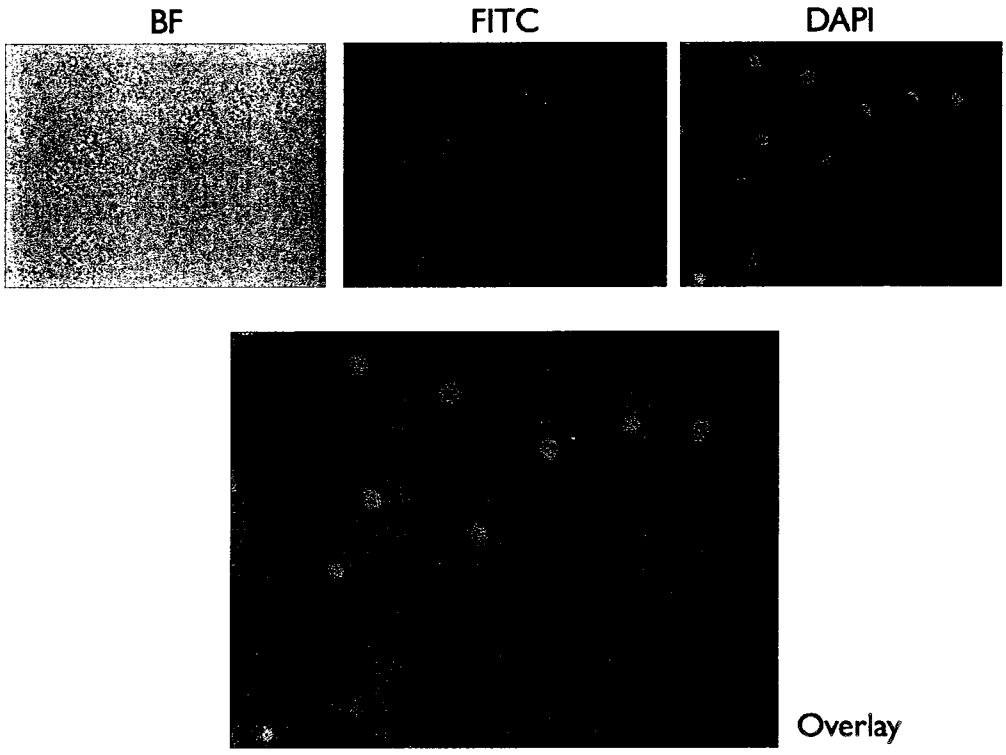
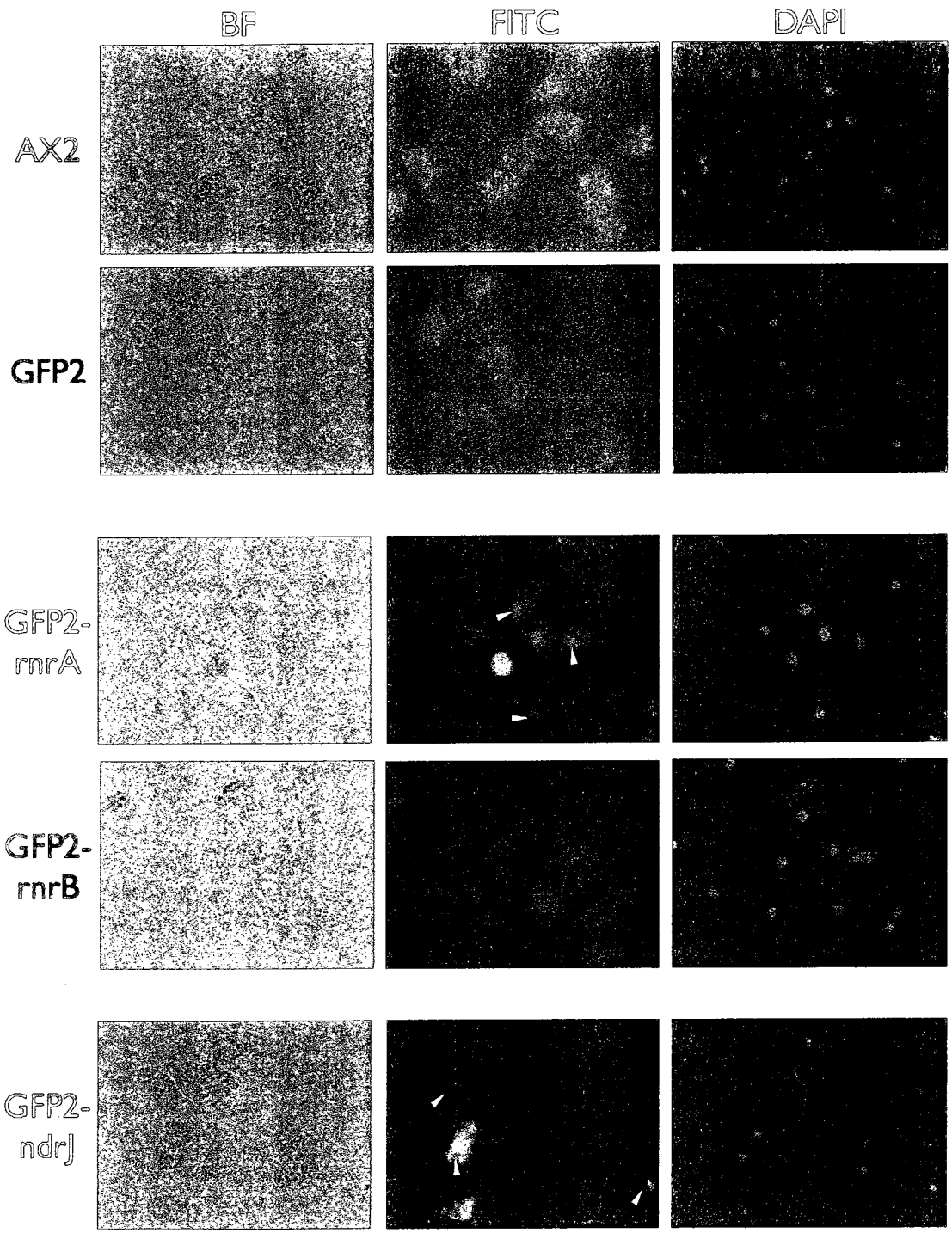


Figure 23: RnrB is not localized to the nucleus or the mitochondria

Images show GFP2-rnrB subunit after incubation in Loflo+yeast and fixed with ethanol. The DAPI stains DNA present in the cell, including the mitochondria. An overlay between the FITC image and DAPI image shows that the GFP2-rnrB is not localized to the nucleus or the mitochondria.

Figure 24: Localisation of RNRs in early development

Cells were starved for 4 hours before ethanol fixation and imaging. GFP2-rnrA localizes to the nucleus in the same manner as in growing cells, while GFP2-rnrB is found in small foci in the cytoplasm. GFP2-ndrJ is found in the cytoplasm. White arrows point to the nucleus.


BF
FITC
DAPI
AX2
GFP2
GFP2-rnrA
GFP2-rnrB
GFP2-ndrJ

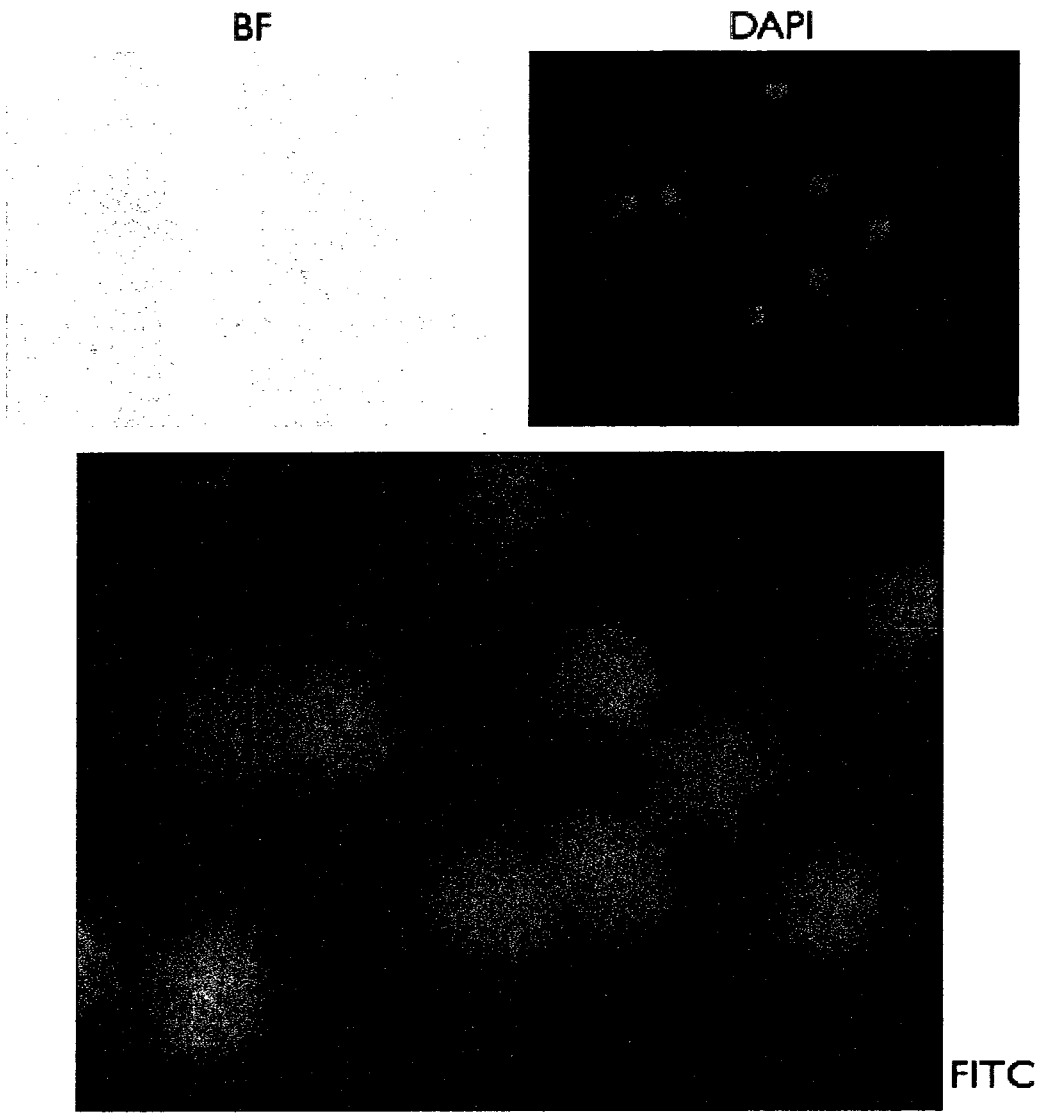


Figure 25: RnrB displays a granular pattern in early development

Images show GFP2-rnrB after incubation in Loflo+yeast and fixed with ethanol. The DAPI stains DNA present in the cell, including the mitochondria. The RnrB localizes in small, diffuse granules in the entire cell.

4 Discussion

4.1 *ndrJ* is not essential for growth and development

Dictyostelium has two complete ribonucleotide reductases from two different classes; the genes *rnrA* and *rnrB* together makes up a Class I enzyme while *ndrJ* encodes a Class II RNR. This is an unusual combination for a eukaryote, where a Class I enzyme is the most common (reviewed by Nordlund, 2006). Class II enzymes are only found in a few eukaryotes (Lundin, 2005). To study whether all the RNR encoding genes are necessary for the organism, we constructed a set of gene replacement plasmids and introduced them to *Dictyostelium* cells. Cells carrying a null allele for *ndrJ,* Δ*ndrJ*::bsR were successfully obtained, showing that the gene is not essential for the organism.

When studying the effect of the gene replacement on growing cells, the two independent gene replacement strains obtained exhibited different generation times (Figure 20). This makes it difficult to draw any definite conclusions about the function of the gene, since the the Δ*ndrJ*::bsR no.1 shows a near-to-normal generation time of 12 hours, whereas Δ*ndrJ*::bsR no.2 displays a generation time of 25 hours. There is no good explanation for this difference, but there is a possibility that the gene disruption affected an important function within the cell. Screening additional mutants could help resolve this issue.

Attempts to replace *rnrB* were also done, but only a few transformants were obtained. These cell lines were resistant to bsr, but were wild type for *rnrB.* The low number of transformants attained makes it hard to draw any conclusions about the

necessity of *rnrB*. It is possible that this gene is essential and in this case the function of the gene will have to be examined using an alternative method such as conditionally expressed promoter to direct its expression, for example the Tet-On or Tet-Off system. These methods will enable for conditional gene expression or gene silencing.

4.2 The RblA protein regulates *rnrA* and *rnrB* through different mechanisms

The transcriptional regulation of the RNRs is complex and initiated under several different cellular conditions. In many systems this is done throughout the cell cycle by the developmental regulator pRB, both through the E2F transcription factor and other, unknown factors (reviewed by Taya, 1997). *Dictyostelium* has a pRB orthologue, RblA. While its function in the cell cycle is unknown, knocking out the gene leads to faster development (MacWilliams, 2006) in addition to increased expression of *rnrA, rnrB* and several other S-phase genes (K. Straβer, personal communication).

Over-expressing the *rblA* in *Dictyostelium* induces a long G1-phase, suggesting a role as a repressor of S-phase genes similar to that found in other organisms. Surprisingly then, *rblA* is mainly expressed in the prespore region of developing cells (MacWilliams, 2006), the same cell type where *rnrB* expression is found (Tsang, 1996). The expression pattern of *rblA* might not seem as surprising when the quiescent state of the prestalk cells are taken into consideration. The prestalk cells have stopped dividing and *rblA* expression may not be required to regulate this process. The expression of *rblA* in the prespore cells might be taken as an indication that these cells are still dividing.

To examine where in the developing structures the RNRs are expressed, a set of reporter vectors were constructed where β-galactosidase is expressed under the control of the different RNR promoters. Like *rnrB*, *rnrA* was found to be expressed exclusively in prespore cells (see Figure 21 b and d).

The reporter constructs were also transformed into the *rblA*-null cells to examine whether the expression pattern of the RNR changed in these cells in addition to the increased expression. While the *rnrB* expression remains exclusive to the prespore cells, the *rnrA* loses its cell-type specificity in the *rblA*-null strain and is expressed in the entire structure (see Figure 21 c and e). In developing *rblA*-null cells, the promoter activity of both *rnrA* and *rnrB* are increased compared to wild type cells. Taken together, these results suggest that RblA regulates *rnrA* and *rnrB* via different mechanisms. A structural search of the upstream region of the *rnrA* coding region revealed two potential E2F binding sites (see Figure 26 for schematic representation of promoter region). These sites matche the E2F consensus sequence, TTTSSCGC (Lincker, 2004) and are oriented in the same manner as the E2F sites of the RNR small subunit in tobacco (Chabouté, 2000). E2F regulates RNR transcription in tobacco, both during the cell cycle and upon DNA damage (Lincker, 2004). E2F has also been shown to activate RNR transcription in tobacco and *Arabidopsis* in S-phase, while it acts as a repressor when DNA synthesis is not necessary (Chabouté, 2000). In human and mouse, E2F serves as an inhibitor to transcription of both RNR subunits during the cell cycle, but direct binding between E2F and the enhancer has only been shown for the small subunits (Angus, 2002; Chabes, 2004; Thelander, 2007). The presence of putative E2F binding sites in the promoter region of *rnrA* suggests that the RblA protein regulates *rnrA* expression via E2F.

Moreover, the absence of putative binding sites for E2F in the promoter region of *rnrB* suggests that RblA regulates *rnrB* via factors that are distinct from E2F. This hypothesis helps to explain why the spatial pattern of expression of *rnrA* and *rnrB* is different in the *rblA*-null strain. It is possible to examine whether the E2F binds to the *rnrA* promoter through an electrophoretic Mobility Shift Assay with E2F. For a different approach, it is possible to tag the RblA protein with for instance a TAP-tag. Gently purifying the protein using a column followed by SDS-PAGE and mass spectrometry can potentially give an indication as to which factors are associated with the RblA protein.

An attempt to map out the expression pattern of *ndrJ* using β-galactosidase as a marker was also tried (see Figure 21 f and g), but no signal was detected following the histochemical staining of cells expressing lacZ under the *ndrJ* promoter. Expression of *ndrJ* is known to be low in developing cells (M. Crona, personal communication) and this could be the reason for the lack of detectable signal. According to an microarray study, *ndrJ* is down-regulated in the *rblA*-null strain and this indicates that *ndrJ* is regulated in a different manner than *rnrA* and *rnrB* which are both up-regulated (K. Straβer, personal communication).

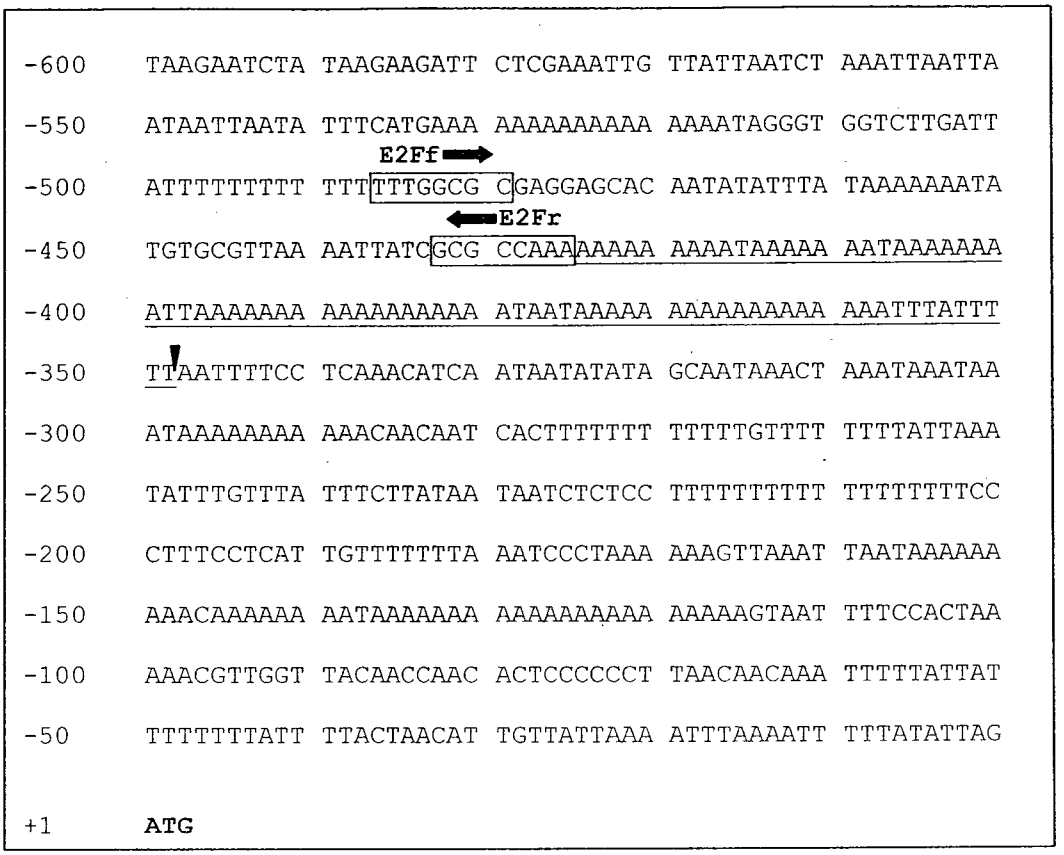


Figure 26: Schematic presentation of the upstream region of *rnrA*.

Boxed regions represent the two E2F consensus sequences (forward and reverse). Underlined region is A/T rich and a vertical arrow represents possible transcription start site (based on EST sequences). The region PCR amplified and cloned to construct the plasmid rnrA-ile-αgal encompasses this entire region.

4.2.1 *rnrB2* is a pseudogene

It is not known whether the second gene encoding a putative small subunit of the Class I enzyme, the *rnrB2* (dictybase gene ID: DDB_G0291), is a functional protein. No ESTs of this gene has been reported (Fey, 2009) and it is not detectable by RNA (Northern) blot hydridization (M. Crona, personal communication) or RNA sequencing using the Illumina/Solexa platform (K. Straβer, personal communication). In addition to this, the coding sequence of *rnrB2* lacks 25% of the carboxyl terminal of the RNR small subunit which suggests that it is incapable of forming the iron centre necessary for proper molecule function (Fey, 2009). We have not been able to detect any activity from the *rnrB2* promoter using β-galactosidase as a reporter. This makes it unlikely that it codes for a functional protein and dictybase.org has removed it from its list of protein encoding genes.

4.3 The RNRs localize to different sub-cellular compartments in *Dictyostelium*

The sub-cellular localisation of the RNR subunits are adapted to different needs during the cell cycle and DNA damage both in yeast (Yao, 2003; Zhang, 2005) and mammals (reviewed by Thelander, 2007). The localisation of a protein can be adapted to where it is needed and spatially segregated when the protein is not required. The sub-cellular localisation of RNR has been used as an indicator of where in the cell ribonucleotide reduction takes place (Thelander, 2007; Yao, 2003; Zhang, 2005). In *Dictyostelium* it is not only unknown where in the cell ribonucleotide reduction takes place, but localizing

this reaction could also elucidate why this organism has two classes of RNR and whether it is mitochondrial or nuclear DNA synthesis that takes place during late development.

Using fluorescent fusion proteins, we have shown that the Class I large subunit, RnrA, is mainly found in the nucleus of the cells (see Figure 22). This stands in contrast to both mammals and yeast where this subunit is localized to the cytoplasm during normal cell cycle progression (Thelander, 2007; Yao, 2003; Zhang, 2005).

In *Dictyostelium* cells, The Class I small subunit, RnrB, is localized in a granular pattern in the cytoplasm during growth (see Figure 22). Similar patterns have previously been reported in yeast (Kumar, 2002; Yao, 2003) and by the RNRs transcribed from the Herpes genome in infected mammalian cells (Conner, 1995). In yeast this pattern was observed upon entry to S-phase or following DNA damage and it is thought that these granules are the seats of active ribonucleotide reduction. During the rest of the yeast cell cycle, the small RNR subunit is sequestered to the nucleus (Yao, 2003). The granules observed in *Dictyostelium* could be seats of RNR activity, and the varying number of granules within the cells could be due to ribonucleotide reduction as a response to different kinds of stressors: S-phase related, mitochondrial DNA synthesis in mid-G2 or DNA repair. This can, however, not be conclusively determined without further research where expression pattern is correlated to cell cycle or DNA damage is induced before RNR localisation is examined.

The RNR localisation was also studied during early development, at a point in the life cycle where the RNRs are usually not expressed; the fusion proteins were expressed under the strong Actin15 promoter, which is known to be active during the first hours of development. Both the GFP2-rnrA and GFP2-ndrJ remained localized in the same

manner as in growth, but interestingly, the GFP2-rnrB changed localisation pattern and the low number of bright granules present in growing cells were replaced with a high number of smaller granules (see Figure 24 and Figure 25).

The Class II enzyme, the NdrJ is found in the cytoplasm with a marked exclusion from the nucleus. This enzyme has been studied to a limited extent and its localization in other eukaryotes is not known.

The results shown here do not elucidate where in the cell ribonucleotide reduction takes place; other methods will have to be used to examine this question. The two subunits of the Class I enzyme can be colocalized by fusing them to different fluorescent proteins before visualising. As an alternative approach, antibodies have been used to localize the RNRs in yeast (Yao, 2003; Zhang, 2005) and mammals (Xue, 2003) and this could potentially be used in *Dictyostelium* as well.

To ensure that ribonucleotide reduction is taking place upon observation, cells can be synchronized so they are at a point in the cell cycle where ribonucleotide reduction is known to take place before fixation and observation. Alternatively, they could be subjected to DNA damaging agents such as UV light, methyl methane sulfonate, cycloheximide or hydroxyurea. This is known to induce RNR transcription and relocalisation in yeast (Yao, 2003) and mammals (Tanaka, 2000), but while the RNR localisation in yeast is the same during S-phase and DNA damage, it changes in mammalian cells dependent on the stressor the cell is subjected to (reviewed by Thelander, 2007), a question that could be examined further also in *Dictyostelium*.

4.4 Class I RNR is the main enzyme in *Dictyostelium*

While any possible combination of the three RNR classes can be found in bacteria, the consensus in eukaryotes is genes encoding for a single Class I enzyme (Nordlund, 2006; Torrents, 2002). Thus, *Dictyostelium* makes an exception having both a Class I and a Class II enzyme. In facultative anaerobic bacteria it is thought that the different combinations of RNRs will enable them to switch between enzymes depending on the conditions (Nordlund, 2006), but *Dictyostelium* is a strict aerobe (Srinivas, 1980) and does not seem to require an oxygen independent enzyme.

It is thought that an organism can obtain several enzymes carrying out the same function either through lateral gene transfer or through duplication of an excising gene that later becomes modified into a new enzyme (Torrents, 2002). It is possible that the first eukaryote derived genes encoding two or three classes of RNRs from its prokaryote ancestor. These enzymes could have been lost gradually as the eukaryote became more adapted for aerobic metabolism (Torrents, 2002). *Dictyostelium* could be an exception to this and retained both enzymes. The *Dictyostelium* Class II enzyme is closely related to the one of *Euglena gracilis* and it is thought that they are derived from a common ancestor, rather than through lateral gene transfer from the same host (Torrents, 2005). The root of the eukaryotic tree is thought to lie between the lineages between *Dictyostelium* and *Euglena* and Torrents *et al.* (2005) took this as evidence towards the theory of common ancestry (Torrents, 2005). But while *Euglena* lost its Class I enzyme, *Dictyostelium* retained both.

We have shown that it is possible to construct a functional knock out the *ndrJ* in *Dictyostelium,* which leaves the cell with the Class I enzyme as the functional RNR. In

addition to this, *rnrB2* (dictybase gene ID: DDB_G0291764) has been suggested to be a pseudogene. We started trying to determine whether the different ribonucleotide reductase genes were able to compensate for each other and our results indicate that *Dictyostelium* can manage with a Class I enzyme as most eukaryotes.

5 Conclusions

To examine whether the Class I and Class II enzyme in *Dictyostelium* is able to compensate for each other, disruption of the different RNR genes were attempted. A gene replacement strain of *ndrJ* was successfully constructed. The strain carrying a functional knockout of *ndrJ* developed normally, but seemed to have somewhat slower growth. This indicates that *ndrJ* is not essential for growth and development in *Dictyostelium* and that the Class I enzyme can compensate for the loss of the Class II enzyme.

rnrB has previously been located to the prespore cells in the developing structures and we found *rnrA* to exhibit the same expression pattern. Both *rnrA* and *rnrB* are upregulated in the *rblA*-null strain, but our results suggest that only *rnrA* also changes expression pattern and loses its cell type specificity.

Localising the subunits of the RNR enzyme could potentially indicate whether the developing cells undergo terminal differentiation as G1 or G2 cells. Our results show that the large subunit of the Class I enzyme is sequestered to the nucleus in growing cells and in early development, while the small subunit is localized in a granular pattern in the cytoplasm. The Class II enzyme is found in the cytoplasm in both growing and developing cells.

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