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Analysis of *cis*-acting elements of the *rnrB* gene of  
*Dictyostelium discoideum*

Abraham Shtevi

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of  
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## ABSTRACT

### Analysis of *cis*-acting elements of the *rnrB* gene of *Dictyostelium discoideum*

Abraham Shtevi

The *rnrB* gene of *Dictyostelium discoideum* codes for the prespore specific small subunit of ribonucleotide reductase. The protein is expressed at two distinct times during the *Dictyostelium discoideum* developmental cycle; first at the onset of development (vegetative activity) and then again during late development at the slug stage (developmental activity). The  $\beta$ -galactosidase gene was used as a reporter gene and was fused to the noncoding area upstream of the *rnrB* gene. A series of 5' and internal deletions were carried out in order to isolate the regulatory regions controlling gene expression. As well, primer extension was carried out on purified mRNA to determine the 5' transcriptional start site of the *rnrB* gene.

Results indicate that vegetative and developmental activities are regulated by separate promoter regions. Vegetative expression was mapped to an A/T rich region spanning -213 to -136. Developmental activity was found to be regulated by two G/C rich boxes, Box A (-238 to -221) and Box B (-313 to -299). Primer extension analysis of the transcript mRNA indicated a single transcriptional start site located 115 bases upstream of the start codon.

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## I. INTRODUCTION

*Dictyostelium discoideum* is a cellular slime mold first isolated from deciduous woods in North Carolina [Raper, 1935]. It has an asexual life cycle with two distinct phases - vegetative and development. During vegetative growth the cells divide through binary fission [Loomis, 1975] with a doubling time of 8-10 hours. Removal of nutrients initiates starvation conditions and the onset of development. *Dictyostelium discoideum* can also reproduce sexually, although this is not an essential phase in its life cycle. A small proportion of cells (1 in 10000) have been observed to engulf one another and become heterokaryotic. The heterokaryons can then either be destroyed or become true diploids [Loomis, 1975].

Development proceeds at a well characterized course beginning with aggregation of individual cells into mounds of up to  $10^5$  cells [Loomis, 1975]. The aggregate forms a finger-like structure with the apex constituting future stalk (or prestalk) cells, and the base comprising prespore cells [Loomis, 1975]. The finger structure goes on to form a sausage-like structure commonly named slug. The slug migrates for several hours and ends up in a culminate. The final arrangement is a mature fruiting body comprised of vacuolated stalk cells supporting a spherical structure composed of viable spore cells. This developmental cycle takes approximately twenty-four hours. The spores remain in the fruiting body until such time as a food source again becomes available.

Figure 1.1.

*Dictyostelium discoideum* developmental cycle. The cycle is triggered by a depletion of the food source and the development from unicellular organism to mature fruiting body takes about 24 hours.

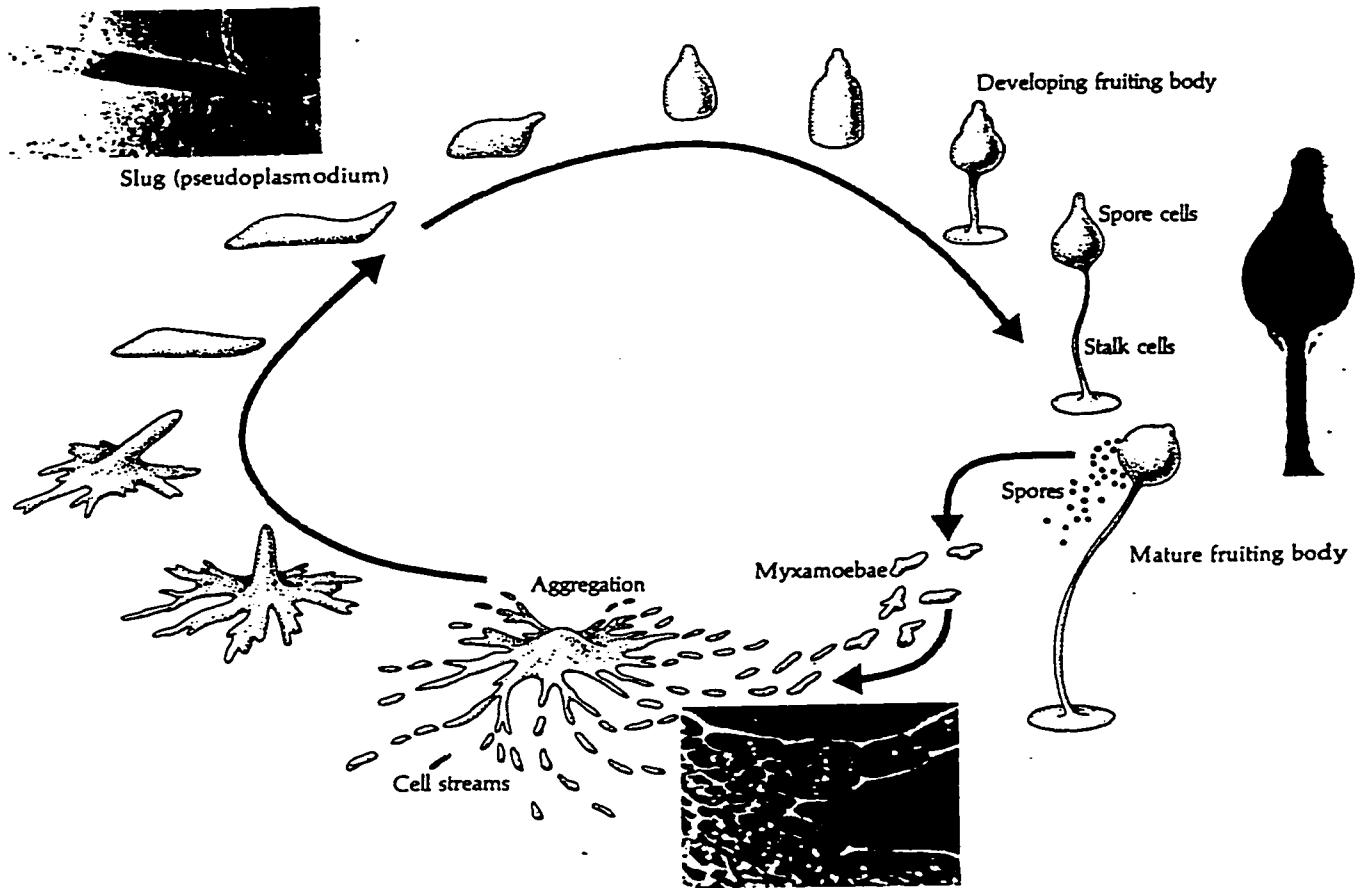


Figure 1.1. *Dictyostelium discoideum* developmental cycle [Gilbert, 1991].

## I.1. Development

### I.1.A. Vegetative to Aggregative Stage

*Dictyostelium discoideum* cells grow vegetatively when cultured on bacteria or, in the case of axenic cells, either bacterial culture or liquid media [Loomis, 1975]. Throughout this growth they secrete PSF (prestarvation factor). As the cell density increases and starvation becomes imminent the levels of PSF reach a threshold value and early developmental genes are triggered [Clarke and Gomer, 1995]. One of the gene products of early development is gp24, an essential adhesion molecule. A threefold mechanism of adhesion exists in *Dictyostelium* with gp24 appearing at the onset of development, followed by gp80, which appears during the aggregation stage of the developmental cycle. The gp80 is replaced by a third adhesion protein, gp95, during the late stages of development. Cells lacking gp24 do not become adhesive and do not undergo development [Knecht et al., 1987].

As well, key genes involved in the cyclic AMP signaling pathway are turned on which prepare the cells for the switch from vegetative to the developmental phase. These genes include *carA*, *gpaB*, *gpbA*, *pdsA*, *dagA*, and *erkB*. The gene products are CAR1, G $\alpha$ 2, G $\beta$ , phosphodiesterase, CRAC, and a MAP kinase respectively, and they are initially synthesized at low levels but upon starvation are pulse-induced by waves of cyclic AMP and their levels increase dramatically [Loomis, 1996].

Once key amino acids are depleted, development is initiated. Autonomous centers begin pulsing waves of cyclic AMP at a rate of once every six minutes [Firtel, 1996]. These waves are propagated from one cell to the next via the signaling pathway. The cells respond to the waves by migrating towards the source of the signal through chemotaxis [Gross, 1994].

The current model of the cyclic AMP signaling pathway operates in the following manner. Cyclic AMP binds to CAR1, the surface cyclic AMP-binding receptor. A G-

protein interacts with CAR1 and exchanges a GDP for a GTP and dissociates into  $G\alpha_2$  and  $\beta\gamma$  subunits. The  $\beta\gamma$  subunit then goes on to bind to the *dagA* gene product, CRAC, an adapter protein. This protein, with the help of ERK2 binds to adenylyl cyclase, which synthesizes cyclic AMP and secretes it from the cell. This passes on the message to neighboring cells which synthesize cyclic AMP in their turn and pass it on [Loomis, 1996].

The cells respond to pulses, rather than to a constant high level of, cyclic AMP [Pitt et al., 1992]. Once cyclic AMP is bound to the cell for about one minute the receptor adapts and is no longer responsive. A secreted phosphodiesterase (PDE), then hydrolyzes the cyclic AMP and allows the receptors to get resensitized. The PDE is regulated transcriptionally by a complex promoter profile and through an inhibitor which senses levels of cyclic AMP [Hall et al., 1993].

Cyclic AMP also acts as a chemoattractant. This is accomplished through the activation of guanylyl cyclase resulting in a cellular increase in cyclic GMP. Mutations in the cyclic GMP pathway result in cells incapable of chemotaxis. The adenylyl cyclase functions normally in these mutants indicating that the two pathways, although both activated by cyclic AMP, are independent [Firtel, 1995].

Cells migrate towards the source of cyclic AMP and continue to stream until a mound of cells is formed. As shown above, cells not only respond to cyclic AMP but, in turn, produce their own for secretion. As the mound is formed more and more cells are in close proximity and the levels of cyclic AMP rise from nanomolar to micromolar. Constant high levels of cyclic AMP inhibit expression of aggregation genes such as *psdA* and *erkB*. At the same time they induce production of *gbfA*, the gene that codes for GBF-- G box factor [Schnitzler et al., 1994]. This is a transcription factor responsible for initiating the next stage in development, namely, the slug stage.

### I.1.B. Slug Stage

Cells in the mound are exposed to constant high levels of cyclic AMP and start producing GBF. This factor mediates the transcription of postaggregative genes such as *lagC*, a cell to cell adhesion factor, and initiates events leading up to expression of cell-type specific genes such as *ecmA*, *ecmB*, and *cotC*. However, GBF itself is not cell-type specific and acts in both prespore and prestalk gene expression. It therefore functions with other transcriptional factors to mediate expression [Schnitzler et al., 1994]. GBF null mutants form a perfectly normal mound structure but then the cells disaggregate due to absence of *lagC* gene product. Cyclic AMP is again produced in a pulsatile fashion and they reaggregate to reform a mound [Schnitzler et al., 1994].

The gene *ecmA* is stalk specific and the gene product, ST430, forms a sheath around the mound, preventing any more cells from attaching to it. The structure is now no longer a loosely assembled group of cells but a complete and cohesive unit comprising all the cells it requires to proceed to culmination and fruiting body formation.

At this stage there is still no cell differentiation but the cells start expressing stalk or spore specific genes and are referred to as prestalk or prespore cells. There are three classes of prestalk cells, PST-A cells which are at the anterior 10% of the slug and express *ecmA* at high levels and PST-O cells, found in the next 10% and express the same gene but at one fifth that amount. As well there are PST-B cells expressing another prestalk specific gene *ecmB*. PST-B cells are present in the center of the apical tip. Production of these cells is repressed by cyclic AMP so they are spatially localized in a region of low cyclic AMP. It is currently believed that the gene product of *tagB*, TagB, exports a certain unknown peptide that acts as a signal to convert some of the initial prestalk cells, termed PST-I, to PST-A and PST-B. When there are a sufficient number of PST-A cells, a signal is relayed to the PST-I cells to convert the remainder to PST-O. TagC is also present and performs a similar role. Both TagB and TagC are necessary for the proper development of both PST-A and PST-O cell types. Another kind of prestalk-like cell is found dispersed throughout the



prespore cell mass. These are termed ALC or Anterior-Like Cells and express *ecmA* and *ecmB* prestalk markers. These are thought to have a regulatory role in the slug and, if the prespore region is removed from the slug, the ALCs will reform a new anterior tip in the prespore mass [Loomis, 1996].

Transcription of prespore genes is controlled by several factors. Mutations in a PST-I specific gene *carB* results in a predominance of prespore cells. The *carB* gene product, CAR2, is a cyclic AMP receptor and is probably regulating levels of prespore genes in a negative manner by preventing the conversion of PST-I to prespore cells. The enzyme PKA plays an important role in the induction of prespore genes. It must be present and active for GBF to bind to the G-box of several spore coat genes [Loomis, 1996].

### I.1.C. Cell Proportioning

Cell proportioning in *Dictyostelium discoideum* is size independent. This means that no matter the size of the slug, the ratio of prespore to prestalk cells will be maintained at 4:1. It is thought that an inhibitor is produced and secreted from the posterior to the anterior tip to prevent cells switching from prestalk to prespore. This would account for the observation that when prespore cells are surgically removed from the slug, it goes on to convert prestalk cells until such time as the proper ratio is reestablished. It is now believed that initial cell choice is dependent upon the position of a given cell in the cell cycle at the time of starvation. Cells in S and early G<sub>2</sub> go on to form prestalk cells while cells in late G<sub>2</sub> become prespore cells. *Dictyostelium discoideum* cells do not go through G<sub>1</sub>. Cells growing synchronously do still form the proper 4:1 ratio. This is thought to be accomplished through small differences in cell mass and positioning in the cell cycle. Cell plasticity allows the slug mass to quickly alter cell types in instances where part of the slug is disrupted. Cells can alternate between prespore and prestalk cell types throughout development until culmination when terminal differentiation occurs [Loomis, 1993].

#### I.1.D. Culmination Stage

Culmination can be initiated by overhead light. This causes a rapid activation of phospholipase D and an induction of 1,2-diacylglycerol. This second messenger remains at high levels throughout stalk formation and is thought to be involved in regulating the culmination stage [Loomis, 1996].

DIF-1 is a putative morphogen involved in terminal differentiation of prestalk cells. It is not required for the establishment of prestalk cells, but does affect the conversion of PST-I cells to PST-A and PST-O cells [Shaulsky and Loomis, 1996]. The molecule is initially present at low levels during early development but levels start to rise when tipped aggregates are formed. Expression reaches maximal levels in the slug stage and during culmination [Loomis, 1993]. Even though there is an posterior to anterior gradient of the compound it induces prestalk genes at the anterior tip of the slug. It is believed that the DIF-1 is sequestered in the cells and the low pH at the tip releases it. Further evidence to support this idea is the finding that adding ammonia, a base, delays culmination and causes the slugs to migrate for days [Gross, 1994].

PST-B cells are the first to vacuolize and form stalk cells by creating a spiraling cylinder downward thereby pushing the prespore mass upwards. The PST-B cells proceed to the base of the stalk. DIF-1 induces the expression of *ecmB* in PST-A cells and they follow PST-B cells and form the remainder of the stalk. ALCs form the upper and lower cups of the sorus (sporehead) as well as the basal disc.

Halfway through stalk formation *stkA*, a gene expressing a putative transcriptional activator, gets switched on and establishes the pathway of spore cells terminal differentiation. The *stkA* gene is absolutely required for spore formation and in *stkA* null cells prespore cells are converted to stalk cells. It is this gene and a phosphorylated, activated PKA that are required for the induction of the *spiA* gene product. *spiA* is involved in spore encapsulation and is initiated at the top of the sorus and a wave of expression works its way down [Loomis, 1996].

The genes controlling culmination include *gskA*, *carD*, and *dhkA*. *gskA*, encoding glycogen synthase kinase 3, null mutants express predominantly PST-B cells and far fewer prespore cells which results in a stalky phenotype [Harwood et al., 1995]. *carD*, encoding another cyclic AMP receptor CAR4, mutants seem to have the opposite phenotype and are composed mostly of prespore cells with short stalks. It is possible that this gene regulates the activity of glycogen synthase kinase 3 as levels of *ecmB* (the PST-B marker) are greatly reduced. The third gene, *dhkA*, encodes a protein kinase and is believed to repress the expression of *ecmO* in PST-A cells and induce prespore encapsulation as mutants possess weaker stalks and very few spores [Loomis, 1996].

#### I.1.E. DNA Synthesis During Development

*Dictyostelium discoideum* has a life cycle that is known to be composed of two parts, as seen above. Initially it was thought that DNA synthesis, mitosis, and cell division occur only during vegetative growth and are absent during development. However, when the cells were labeled with [*methyl*-<sup>3</sup>H]thymidine and allowed to develop, DNA was found to be synthesized at the onset of development and during post-aggregation. 5-Fluorodeoxyuridine, a drug known to inhibit thymidylate synthase, a key enzyme in DNA synthesis, arrested development at the slug stage. Removal of the drug led to the completion of development and fruiting body formation [Zada-Hames and Ashworth, 1978]. The study suggested that DNA synthesis at the slug stage is necessary for completion of the developmental cycle.

Another study identified the site of DNA synthesis in developing cells. *Dictyostelium discoideum* cells were treated with <sup>3</sup>H-adenine which radiolabeled the DNA. Sites of DNA synthesis were found to be at the prestalk prespore border of developing slugs [Durstun and Vork, 1978].

Later studies focusing on DNA synthesis during slime mold development have attempted to investigate and explain this phenomenon. Nocodazole, a tubulin inhibitor was

used to inhibit mitosis in *Dictyostelium discoideum* [Cappuccinelli et al., 1979]. The results indicated that mitosis was not necessary for fruiting body formation. However, the developmental cycle was affected by the drug and terminal differentiation took longer with increasing doses. Another study, used a different mitotic inhibitor (CIPC) and found that DNA synthesis occurred and the developing cells became polyploid [Weijer et al., 1984]. Later studies using DNA synthesis inhibitors had only marginal success with the drugs that were used, hydroxyurea and aphidicolin. Hydroxyurea blocked spore maturation and lead to the formation of stalky fruiting bodies but had little effect on DNA synthesis. Aphidicolin inhibited DNA synthesis only slightly and had no effect on fruiting body structure and formation [Zimmerman and Weijer, 1993].

There is further evidence that prespore cells do undergo mitosis. A fluorometric assay was used to quantify the amount of DNA in prespore cells [Sharpe et al., 1984]. It was found that while cells in mid-G<sub>2</sub> did go on to form spore cells DNA content in spore cells was low compared to control cells ( $0.16 \pm 0.006$  pg/cell compared to  $0.45 \pm 0.021$  pg/cell). It was concluded from this data that cell division has occurred in the prespore cells.

Researchers also focused on the site of DNA synthesis in the developing cells. Labeled DNA was separated through cesium chloride gradients [Deering, 1982]. Nuclear DNA had a different density than mitochondrial DNA and could be separated from it. It was found that the nuclear DNA was getting labeled during post-aggregation while labeled dA and dT get incorporated into mitochondrial DNA throughout development.

In a recent study, *Dictyostelium discoideum* cells were labeled with bromodeoxyuridine and developed for varying lengths of time. The nuclei and mitochondria were separated and the DNA was extracted. Dot blots were prepared and were probed with anti- bromodeoxyuridine antibodies. The same blots were probed with a mitochondrial DNA probe and separately with a chromosomal DNA probe. In all cases, it was found that DNA replication observed during development was mitochondrial. The

Figure 1.2.

Electron pushing mechanism for the reduction of a ribonucleotide diphosphate by the enzyme ribonucleotide reductase. The loss of the  $\text{OH}^\cdot$  is carried out via a free radical reaction. The B represents any of the four ribonucleotides.

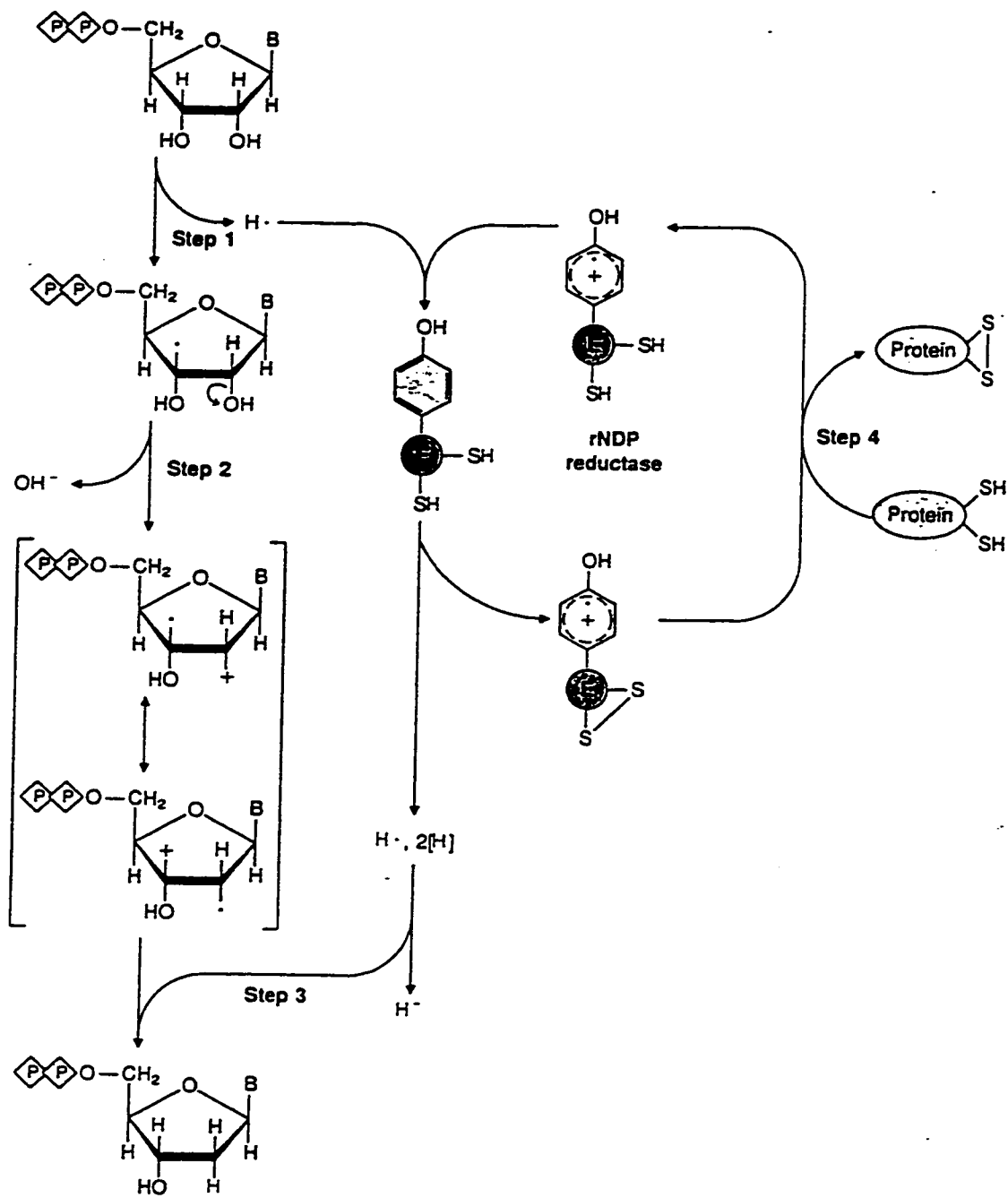


Figure 1.2. Mechanism of the enzyme ribonucleotide reductase [Matthews and Van Holde, 1990].

hypothesis presented is that mitochondrial respiration and elevated energy stores are needed for cells to differentiate into spore cells [Shaulsky and Loomis, 1995].

## **I.2. Ribonucleotide Reductase**

Ribonucleotide reductase is a tetramer composed of two large subunits (RNR1) and two small subunits (RNR2). It is an enzyme directly involved in DNA replication and repair. It catalyzes the reaction  $NTP \longrightarrow dNTP$ ; the reduction of nucleotides into deoxynucleotides. An electron pushing diagram is presented in figure 1.2 and shows the loss of a hydrogen atom from the ribonucleotide creating a free radical. This is followed by an extraction of the OH-group at 2' carbon position. These two losses are then replaced by two hydrogens and the enzyme is subsequently recycled [Reichard, 1997]. This is the first essential step in the de novo synthesis of DNA [Reichard, 1988]. The large subunit seems to be present throughout the cell cycle while the small subunit is produced when needed for DNA synthesis.

While much is known about the structure, function, and mechanism of the enzyme, it has only recently been isolated from *Dictyostelium discoideum* [Tsang et al., 1996]. The gene, *rnrB*, encoding the small subunit of ribonucleotide reductase was found upstream of the *capA* gene and in the opposite orientation. A Northern blot analysis of the *rnrB* transcript during development, shown in figure 1.3, indicated that it was transcribed in the same biphasic manner and at the same times as DNA synthesis. It seemed the ideal tool for investigating DNA synthesis in the organism during development.

## **I.3. Dictyostelium discoideum Promoters**

Over the years many papers have been published dealing with promoter regions in *Dictyostelium discoideum*. These are "composed of modules, thus allowing the definition of elements with specific effects on transcriptional control" [Maniak and Nellen, 1990].

While each gene has a unique promoter profile, certain elements are essential and therefore shared by all, and others are common to many, if not all promoters.

The first element that will be discussed is shared by all genes in all organisms. It is the transcriptional start site. This is the site, upstream of the ATG, where transcription by the RNA polymerase begins. Start sites in *Dictyostelium discoideum* range in distance from 30 base pairs upstream of ATG [Rizzuto et al., 1993] to 380 base pairs [Barklis et al., 1985]. As well, if the message is transcribed at different times during the life cycle or during the developmental cycle, there might be different start sites for the same gene. For example, the gene encoding cytochrome-c oxidase subunit V (COX V), the terminal enzyme of the mitochondrial respiratory chain, has two start sites very close together at -42 and -48 from the ATG [Rizzuto et al., 1993]. These two mRNA species are translated into the same protein with the same activity.

Very often, just upstream of the transcriptional start site is a TATA box. Both this and an oligo(dT) run are common elements in genes transcribed by RNA Polymerase II [McPherson and Singleton, 1993]. TATA boxes are very common as they are thought to bind to a general transcription factor [Struhl, 1987]. In *Dictyostelium discoideum* the consensus sequence is TATAAA [Barklis et al., 1985]. By comparing several such elements it was found that they are between 5-7 bases in length and range from 20 to 40 base pairs upstream from the transcription start site.

As mentioned above, the other element commonly seen in *Dictyostelium discoideum* genes transcribed by RNA Polymerase II is an oligo(dT) run. This is a relatively long stretch of DNA composed almost entirely of Ts and is a feature unique to *Dictyostelium discoideum* [Kimmel and Firtel, 1983]. This element is usually located between the start site and the TATA box and range in size from 10 to 30 base pairs [Kimmel and Firtel, 1983]. In two cases, however, this element was located immediately upstream of the TATA box [Hori and Firtel, 1994]. It has been proposed that the poly(dT)



Figure 1.3.

Northern blot analysis of the *mrB* transcript. Panel A indicates endogenous *mrB* transcript while B shows levels of accumulation of the *mrB/LacZ* transcript. In both cases there is an initial indication of accumulation at the vegetative state which then reappears at the tight aggregate stage (12 hr) and peaks at the slug stage (18 hr).

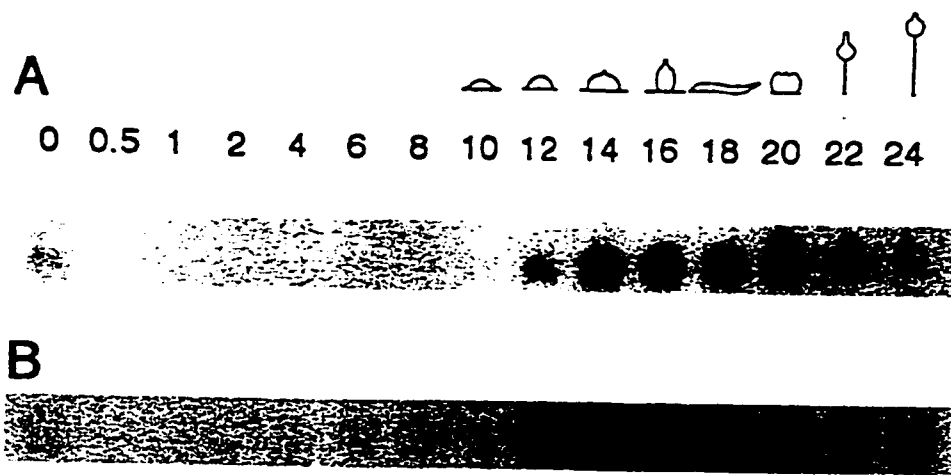


Figure 1.3. Northern blots of *rnrB* and *rnrB/LacZ* fusion [Tsang, 1996].

element controls promoter strength and expression and might direct the binding of TFIIID (transcription factor) to the proper promoter elements [Hori and Firtel, 1994].

*Dictyostelium discoideum* noncoding regions are about 90% A/T [Hori and Firtel, 1994]. Moreover, the organism has a total of about 22% G/C in its genome, making it one of the lowest G/C content organisms known [Pavlovic et al., 1989]. G/C rich pockets could, therefore, be potential sites of regulation. In fact it has been found that many promoters' regulatory elements are composed of CA [Tasaka et al., 1992; Haberstroh et al., 1991] and GT boxes [May et al., 1989; Pavlovic et al., 1989; Rizzuto et al., 1993]. These might be either a random array of Cs and As or Gs and Ts, or it might be a very specific, defined sequence, as in the case of *cotA*, *cotB*, and *cotC* [Tasaka et al., 1992]. These have a CA box of the following sequence (A/T)C(A/T)CCCA. The regulatory sites in the well-characterized *Dictyostelium discoideum* promoters are usually ten to twenty bp long [Kimmel and Firtel, 1983]. Examples of promoters are listed in Table 1.1.

One transcriptional factor promoter known for binding a promoter element is GBF which binds to the G-box. G-boxes are typically found in promoters of genes being expressed after aggregation, for example spore coat protein SP60 [Haberstroh et al., 1991] and SP70 [Fosnaugh and Loomis, 1993]. These G-boxes have varying structures with the only commonality being their high G content.

Sometimes, these C/G rich domains are flanked by palindromes. An example of this is the *pst-cathepsin* promoter region [Datta and Firtel, 1993] with the sequence-AACACAGCGGGTGTGTT (the palindrome is underlined). One possible reason behind this is that the DNA strands might bend and allow a trans-acting factor better access to the promoter element.

Although noncoding DNA is composed almost exclusively of As and Ts, there are a couple of studies to date that claim that A/T elements are the critical regulatory factors. The UDPGP1 gene has a 40 base pair region at 337 bases upstream from the ATG that is A/T rich and is thought to act as a regulatory element, in conjunction with a cyclic AMP

response element upstream, during vegetative growth [Pavlovic et al., 1989]. The *act15* A/T element is 12 base pairs long and is situated 171 base pairs upstream of ATG. Deletion of the 12 base pairs resulted in a 50 fold decrease in luciferase activity in a transient expression system and a 20 fold decrease in a stable expression system. It is not known why these genes use A/T rich regions as critical promoter sites.

Promoters regulate the temporal and spatial activity of genes. The elements discussed above function together to ensure that the right genes are expressed at the right time and at the correct sites in the mound, slug, or culminant structure. Their functioning is essential to the proper completion of the developmental cycle.

Table 1.1.

*Cis*-regulatory elements of several *Dictyostelium discoideum* genes. The elements range in size from 10 to 20 bp and are all G/C rich. Certain cell type specific elements share a common motif, for example the prespore specific boxes of *SP60*, *SP70*, *SP96* and *Dp87* all have a '(A/T)C(A/T)CCCA' motif, indicating that the same trans-acting factor might bind to these elements [Tasaka et al., 1992]. As well, the prestalk specific *ras* and *pst-cathepsin* elements share an 'AACACA' box. These common elements seem to indicate coordinated regulation of the genes during development.

Table 1.1. Various *Dictyostelium discoideum* Promoters.

Gene	cell-type specificity	Promoter sequence	Reference
<i>SP60</i>	prespore	TTCACACACCCACACACTA TTCACACATTAACACACTT AACACACTCCCAACACACAA	Haberstroh, 1991
A11H2		AAAGGGTTT TAGGGGTGGGGGTTTT	May, 1989
UDPGP1		CTACACCCCAAATGGGGTAGTTG AATACCACAAAAGTAGTATT	Pavlovic, 1989
<i>ras</i>	prestalk	AACACAC TGACGGTA	Louvion, 1991
<i>SP96</i>	prespore	TTAAACACCCCAAATT	Tasaka, 1992
<i>SP70</i>	prespore	TCTCTCACCCACACA TCATACACCCACCAA ACCTACACCCAGTTC	Tasaka, 1992
COX V		TGTGGATAAC AATCCCTATC AATATGGATA GGGAATT	Rizzuto, 1993
<i>Dp87</i>	prespore	TCATTACCCACAAT ATATACACCCTAGAC ACTAACACCCATTT	Tasaka, 1992
<i>pst-cathepsin</i>	prestalk	AACACAGCGGGTGTGTT	Datta and Firtel, 1988

## II. MATERIALS AND METHODS

### II.1. Strains and growth conditions

For the generation of the pDdRNR/LacZ construct as well as the deletion set, XL1 Blue and DH5 $\alpha$ F' strains of *Escherichia coli* were used. The cells were maintained either on 1.5% agar plates with LB medium, or in liquid LB [Sambrooke et al., 1989]. Following transformation the cells were cultured in the above medium supplemented with 100  $\mu$ g/ml of ampicillin.

The *Dictyostelium discoideum* strain AX2 was used exclusively [Tsang et al., 1996]. This is an axenic strain and can be cultured either in liquid media (HL5) [Watts and Ashworth, 1970] or on a bacterial lawn of *Enterobacter aerogenes* on SM plates [Sussman, 1966]. The cells were grown at 22°C - 25°C in a moist environment when on plates. Stocks were kept in HL5, 10% dimethyl sulfoxide (DMSO) at -80°C. Transformed cells were cultured in HL5 supplemented with 15  $\mu$ g/ml of G418.

### II.2. Transformation of *Escherichia coli* cells

*Escherichia coli* electrocompetent cells (provided by Claire Bonfils) were removed from the freezer and allowed to thaw. A volume of 20  $\mu$ l of thawed cells were pipetted into a chilled 0.1 cm electroporation cuvette and 1-5  $\mu$ l of DNA were added to the cells. The cuvette was placed on ice for 1 minute and then placed into the Biorad electroporator

chamber. The gene pulser was set to 25  $\mu$ F and 1.25 kV while the pulse controller was set to a resistance of 200 $\Omega$ .

The cells were pulsed and 1 ml of SOC (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose) was immediately added to the cuvette. The cells were transferred to a test tube and shaken at 250 RPM, 37°C for 1 hour. Finally, 200  $\mu$ l of cells were spread on LB plates containing 100  $\mu$ g/ml ampicillin and grown overnight at 37°C.

### **II.3. *Dictyostelium discoideum* growth and transformation**

*Dictyostelium discoideum* was transformed as follows: the cells were grown at 22°C - 25°C in HL5 medium to a density of approximately 3x10<sup>6</sup> cells/ml. Ten milliliters of cells were placed on sterile petri dishes and allowed to rest for 30 minutes. The medium was removed from the plates (the cells adhere to the bottom of the plastic plate) and replaced with 10 ml of MOPS medium (5 g of yeast extract, 10 g of proteose peptone, 10 g of D-glucose, 1.3 g of MOPS, pH 7.1 in one litre). Thirty minutes later the MOPS medium was removed and 600  $\mu$ l HBS (0.137 M NaCl, 4.83 mM KCl, 0.704 mM Na<sub>2</sub>HPO<sub>4</sub>, 21 mM hepes, 5.55 mM dextrose, pH 7.05) with 50  $\mu$ g of DNA and 125 mM CaCl<sub>2</sub> was added dropwise to the cells. MOPS medium was added to the cells thirty minutes later and the cells were left at room temperature for 5-6 hours. Following 5-6 hours, the medium was removed and the cells were treated with 2.5 ml of 18% glycerol in HBS. Five minutes later the glycerol solution was removed and 10 ml of HL5 medium was added. The plates were incubated overnight at 22°C in a moist chamber. The next day the medium was



replaced with fresh HL5 medium containing 15 µg/ml G418. The cells were incubated at room temperature in the moist chamber and the medium with the G418 was replaced every 2-3 days. Colonies appeared usually after one week of selection [Nellen et al., 1987].

The colonies were pooled and transferred to 125 ml flasks containing 25 ml HL5 medium with G418 (15 µg/ml). The flasks were shaken at room temperature. The cells were subcultured every two-three days.

To initiate development, the cells were grown until they reached a density of  $1-2 \times 10^6$  cells/ml. They were then centrifuged at 400g for 5 minutes and washed with KK2 buffer (14.7 mM  $\text{KH}_2\text{PO}_4$ , 2 mM  $\text{Na}_2\text{HPO}_4$  pH 6) once, and resuspended in KK2. The cells were plated on 1.5% agar containing KK2 at  $1.76 \times 10^6$  cells/cm<sup>2</sup>. The plates were kept in a moist chamber at 23°C.

## II.4. Vector and Deletion Constructs

### II.4.1. Generation of pDdRNR/LacZ

Two plasmids were used to construct pDdRNR/LacZ. The first was the pDdGal16 vector [Harwood and Drury, 1990]. It is a convenient shuttle vector containing the ampicillin resistance gene and neomycin resistance gene. As well, it contains the functional segment of the *lacZ* gene. The multiple cloning site is immediately upstream of the *lacZ* sequence and allows in-frame insertions of coding DNA. There are three pDdGal vectors available and pDdGal16 was chosen as the *lacZ* gene reading frame was not affected by the insertion of the *XhoII/BamHI* insert.

The second plasmid used was pDdM100, a previously constructed plasmid containing the 5' end of *rnrB*, encoding the small subunit of ribonucleotide reductase, a

segment of the *capA* gene and a stretch of noncoding DNA between the two. The promoter was assumed to be situated somewhere in the noncoding region.

The pDdGal16 vector was linearized with *Bg*III and dephosphorylated. The 2.7 kilobase *Xho*II/*Bam*HI fragment from pDdM100 was ligated to the vector as the cohesive ends were compatible. The construct was named pDdRNR/LacZ, and it would lead to the expression of a ribonucleotide reductase/ $\beta$ -galactosidase fusion protein in *Dictyostelium discoideum*. The three plasmids are shown in figure 2.1.

Figure 2.1.

The plasmids used in the study. Figure 2.1.1. shows the plasmid pDdM100. It is a Blue Script+ vector containing a 4 kb genomic fragment from *Dictyostelium discoideum* composed of the *capA* gene, noncoding sequence and a segment of the *rrnB* gene. Figure 2.1.2. is the map of pDdGal16, the shuttle vector used. The multiple cloning site lies directly upstream of the *lacZ* gene. The star indicates a restriction cut site that is not unique. Figure 2.1.3. is a map of pDdRNR/LacZ. It is composed of a 2.7 kb *Xho*II to *Bam*HI section of the genomic fragment still containing a part of *capA*, the entire noncoding region, and most of the *rrnB* gene fused to the *Bgl*III site of pDdGal16. This construct was used to generate all the deletions described in the text.

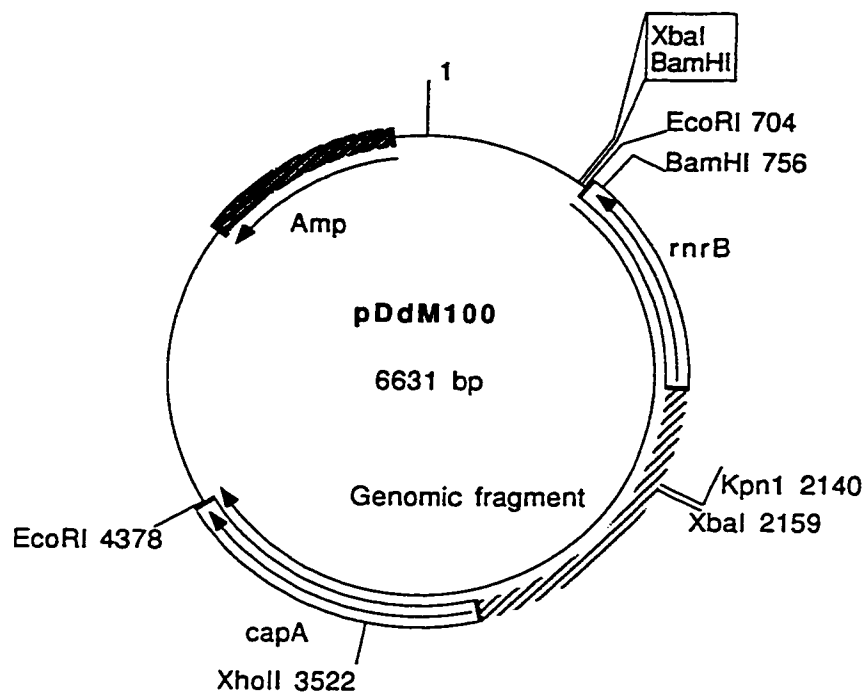


Figure 2.1.1. Plasmid map of pDdM100.

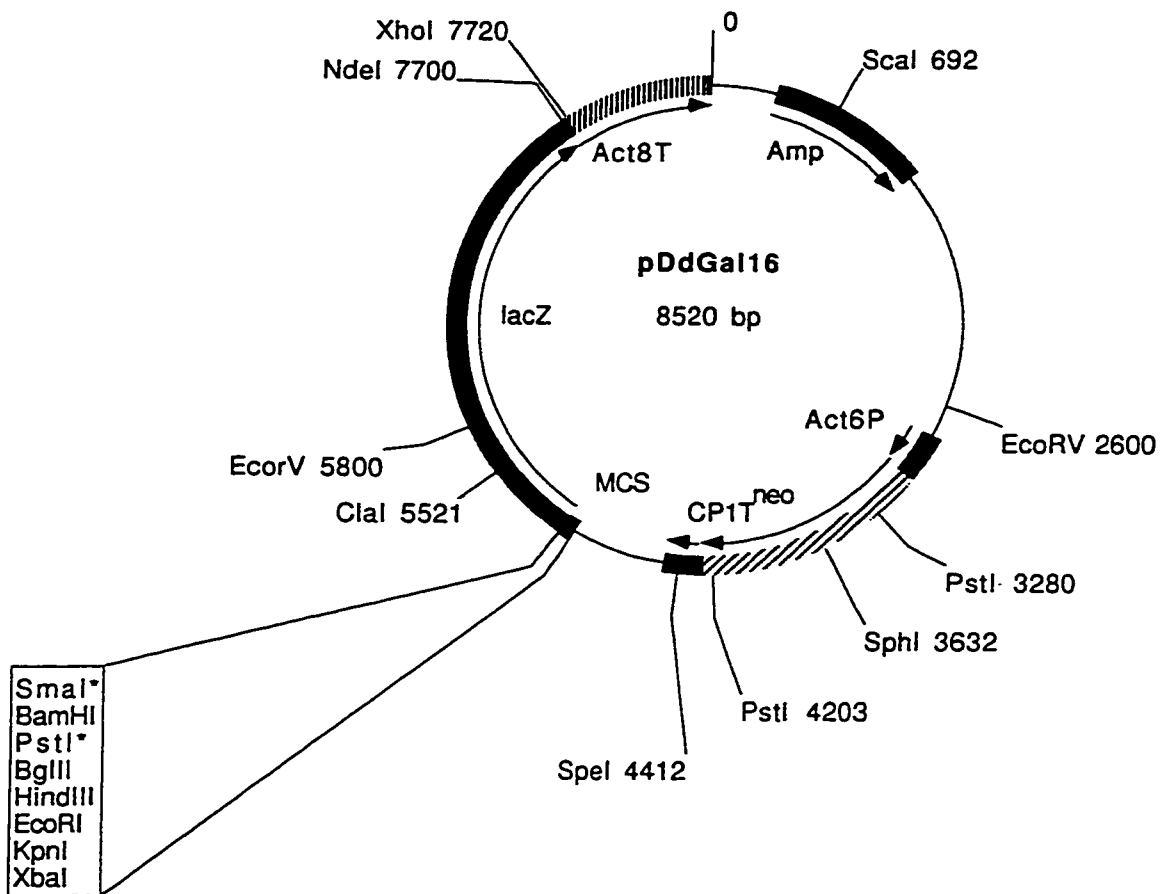


Figure 2.1.2. Plasmid map of pDdGal-16 [Harwood and Drury, 1990].

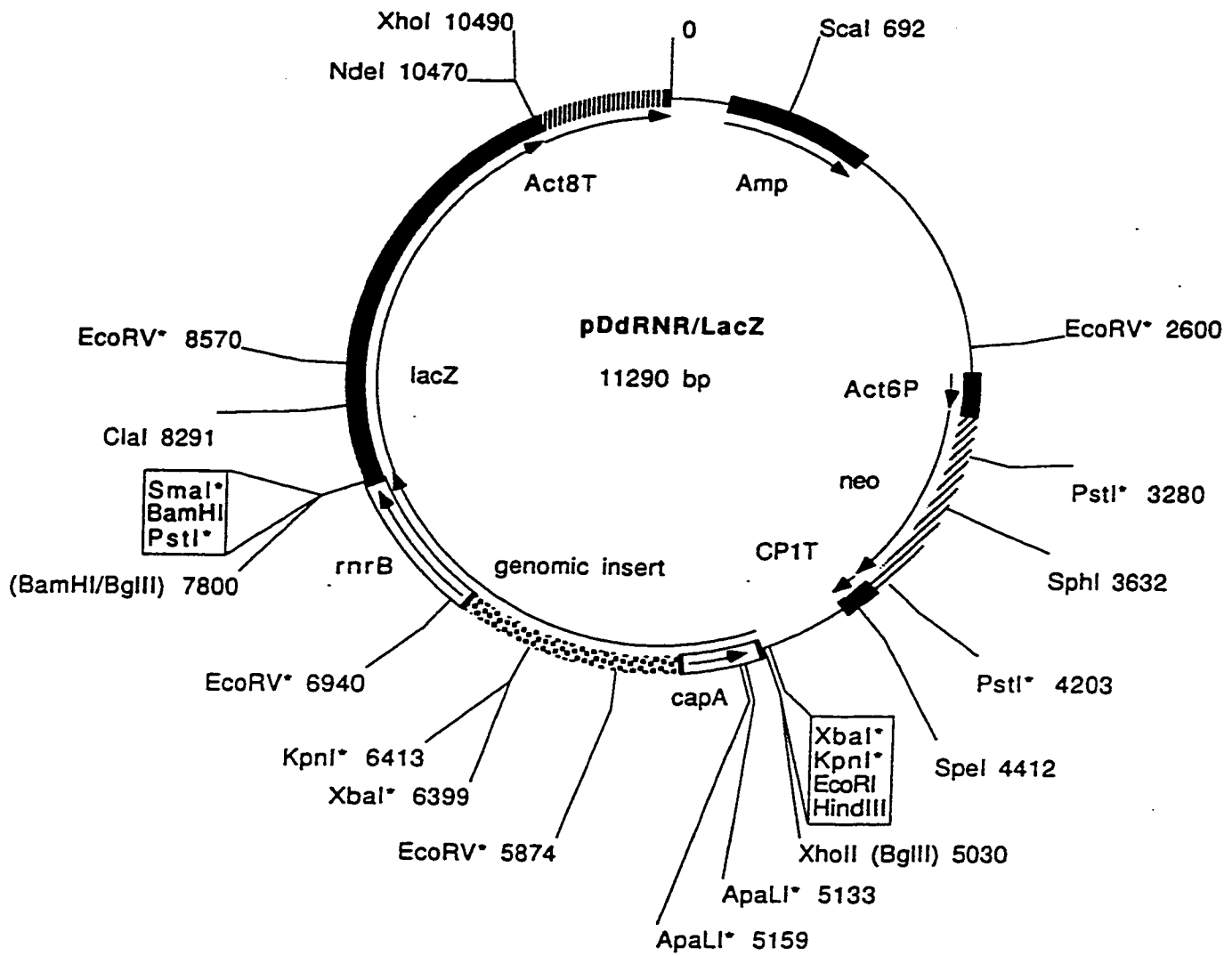


Figure 2.1.3. Plasmid map of pDdRNR/LacZ.

#### II.4.2. Construction of *KpnI*, *XbaI*, and *DraI* deletions

A few internal deletions could be generated by simple restriction enzyme cleavage and religation. This is due to the fact that for the *KpnI* and *XbaI* deletions, there were two sites on the parent construct; one in the multiple cloning site and the other in the 5' noncoding region, 449 bases (*XbaI*) and 430 bases (*KpnI*) from the ATG.

*DraI* had to be constructed in a different way as there are several *DraI* sites in the noncoding region. The construct pDdRNR/LacZ was digested with *DraI* and *BamHI*. As *DraI* is a blunt-end cutter, the pDdGal16 vector was digested with *EcoRI* and blunted with Klenow. The linearized plasmid was then digested with *BamHI*, and ligated to the *DraI/BamHI* fragment purified from pDdRNR/LacZ [Sambrooke et al., 1989].

#### II.4.3. Generation of random deletions using DNase I

DNase I was used to generate random deletions spanning the length of the noncoding region [Sambrooke et al., 1989]. The pDdRNR/LacZ construct was linearized at random positions using 1.5 µg of 1 µg/ml DNase I in 0.01 M HCl for 1 minute, and then cut a second time with *EcoRI*. The deletions were then ligated together. Dnase I cuts the plasmid randomly, while the *EcoRI* only cuts in the multiple cloning site. The desired deletions would eliminate the multiple cloning site downstream of *EcoRI* as well as a variable length of the cloned insert. The ligation products were then digested with *HindIII*, a restriction site downstream of *EcoRI* that should not be present in the desired deletions. Analysis of approximately six hundred deletions yielded about 10 putative deletions and of those only three were shown to be in the noncoding region. A different strategy using Exonuclease III was attempted.

#### II.4.4. Generation of random deletions using Exonuclease III

Exonuclease III digests were carried out using a modification of the Erase-A-Base kit (Promega). The pDdRNR/LacZ vector was linearized with *EcoRI*. The exonuclease III

digests linearized DNA in a 3'—→ 5' fashion (It does this at a controlled rate which is temperature dependent with a higher incubation temperature corresponding to a faster digestion rate). Due to its mode of digestion, the enzyme created long strands of single stranded DNA. The digested samples were then incubated with S1 nuclease. The S1 nuclease digested away the single-stranded DNA. The shortened inserts were cleaved from the digested construct with *Bam*HI and religated to pDdGal16 that had been linearized with *Hind*III and *Bg*II. Colony hybridizations were carried out to isolate potential deletions.

## II.5. Colony Hybridizations

To facilitate the identification of deletion constructs, transformants were examined by colony hybridization. Colonies were patched on a plate and grown overnight. They were transferred to 4°C for 2 hours and then lifted onto a nitrocellulose filter, lysed for 3 minutes in 10% SDS, denatured for 5 minutes on 0.5 N NaOH, 1.5 M NaCl, placed in neutralization buffer (1.5 M NaCl, 0.5 M Tris·Cl, pH 7.4) for 5 minutes and finally moved to 2x SSC solution and left there for 20 minutes. The DNA on the filters then crosslinked to the filters using the Stratagene UV Stratalinker™ 1800 at 120 mJoules (auto-crosslink setting).

The DNA used to probe the filter was a 1.4 kb fragment corresponding to the *Xba*I to *Bam*HI segment of the pDdRNR/LacZ. It was cleaved and purified using GeneClean (BIO 101, Inc.). The purified DNA was labeled with Klenow and [ $\alpha$ -<sup>32</sup>P]-dCTP.

The filters were incubated in the hybridization solution (50% formamide, 6.7x SSC, 0.5% SDS, 5x Denhardt's solution, 0.2 ml of 100  $\mu$ g/ml denatured salmon sperm



DNA, 10 ml total volume) for 30 minutes at 37°C. The radiolabeled probe was then added to the solution and the filters were hybridized overnight at 37°C.

The next day the filters were washed in 2x SSC, 0.1% SDS at room temperature for fifteen minutes. Three more washes were then carried out in 1x SSC, 0.1% SDS; the first at 45°C, the second at 55°C, and the last at 64°C. All three washes lasted fifteen minutes. The membranes were then exposed overnight on X-ray films [Sambrooke et al., 1989].

## **II.6. Sizing of deletions**

The deletions obtained through the various methods were then sized on agarose gels. A double digest of *Xba*I and *Eco*RV would yield several bands including a small band ranging in size from 220-550 base pairs, depending on the size of the deletion. While this method would not yield accurate size determinations of the deletions, it would provide an initial assessment of the deletion set. In this way, only deletions that bordered in the noncoding region were characterized further by sequencing.

Sequencing was carried out using the T7 sequencing kit (Promega). Template was prepared using the standard alkali plasmid preparation, further purifying it by a lithium chloride precipitation, followed by a pancreatic RNase digestion. The pRNase was then removed by one round of phenol:chloroform extraction.

The primer used was a 20mer of the following sequence: 5' GAG AAT TGG TTC AAT GAA TG 3' with the 5' end annealing 42 bases downstream from the ATG. The samples were run on a 6% acrylamide gel for 3-5 hours, depending on the estimated size of the deletion obtained from the sizing done by agarose gel analysis.

## II.7. Liquid Assays for $\beta$ -Galactosidase Activity

Cells were collected from plates using KK2 buffer and placed at  $-80^{\circ}\text{C}$ . The cells were lysed by one round of freeze-thaw. The tubes were thawed and spun at  $13800 \times g$  for 10 minutes. The clear supernate kept for further analysis.

Assays for  $\beta$ -galactosidase activity are conducted as follows. Disposable cuvettes were prewarmed in a  $30^{\circ}\text{C}$  water bath.  $300 \mu\text{l}$  of Z buffer (60 mM  $\text{Na}_2\text{HPO}_4$ , 40 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM KCl, 1 mM  $\text{MgSO}_4$ , pH 7) was added to each cuvette,  $400 \mu\text{l}$  in the blank.  $100 \mu\text{l}$  of supernate from the cell lysate was added to the cuvettes (with each sample done in duplicate); nothing was added to the blank.  $232 \mu\text{l}$  of 4 mg/ml ONPG in 50 mM  $\beta$ -mercaptoethanol was added to the cuvettes and the timer was started. The reaction was allowed to proceed until a yellow color appeared in the cuvettes. The reactions were stopped by the addition of  $400 \mu\text{l}$  of 1 M  $\text{Na}_2\text{CO}_3$ , and the elapsed time recorded. The reaction mixtures were diluted with 1 ml of Z buffer and their OD measured at 420 nm [Dingermann et al., 1989].

Specific activity of  $\beta$ -galactosidase was then calculated by first converting the  $\text{OD}_{420}$  reading obtained from the enzyme assay to pmol of *o*-nitrophenol produced. This was calculated using Beer's law  $A = \epsilon bc$  [Harris, 1987], where  $A$  is the  $\text{OD}_{420}$  of the sample;  $\epsilon$  is  $4500 \text{ M}^{-1}\text{cm}^{-1}$ , the molar absorptivity of *o*-nitrophenol [Miller, 1972];  $b$  is 1 cm, the pathlength of the light in the cuvette; and  $c$  is the concentration of the sample in moles/litre. The concentration of *o*-nitrophenol produced in the reaction was then divided by the time of the reaction in seconds. This provided a pkat value for the reaction catalyzed by the  $\beta$ -galactosidase in the sample. Finally, specific activity was determined by dividing pkat of a

given sample by the amount of protein in milligrams present in the reaction. These calculations were done in duplicate and the averages were plotted on a specific activity, in units of pmol/sec/mg protein, versus developmental time in hours.

Protein concentration in the cell lysate was determined by using the Bradford Assay [Bradford, 1976]. 795  $\mu$ l of KK2 buffer was added to the cuvettes. To the buffer, 5  $\mu$ l of supernatant was added. All samples were done in duplicate and the blank cuvette contained 800  $\mu$ l of KK2 buffer. To the diluted protein sample, 200  $\mu$ l of Bradford's reagent (stock solution of 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol, 100 ml of concentrated phosphoric acid, 50 ml of H<sub>2</sub>O) was added. The samples were measured at OD<sub>595</sub>. A calibration curve was constructed using Ovalbumin at a concentration range of 1  $\mu$ g to 25  $\mu$ g.

## **II.8. Primer Extension of Ribonucleotide Reductase RNA Obtained From *Dictyostelium discoideum***

### **II.8.1. Isolation of total RNA from *Dictyostelium discoideum* and preparation of poly(A)<sup>+</sup> RNA**

Poly(A)<sup>+</sup> RNA from *Dictyostelium discoideum* was isolated from approximately 1x10<sup>9</sup> cells. The cells were developed for 17 hours, 10 mM MMS (Methyl Methanesulfonate) in KK2 was added and development was continued for an additional hour. Treatment with MMS increases the levels of *rnrB* transcripts severalfold. The cells were then pelleted and lysed by a freeze-thaw cycle at -80°C. Total RNA was isolated according to the method described by J. Franke [Franke et al., 1987]. The pellets were dissolved in GSEM (50% guanidiniethiocyanate, 0.5% sarkosyl, 25 mM EDTA, pH 7.0;  $\beta$ -mercaptoethanol was added to a final concentration of 0.1% immediately prior to use).

Equal volumes of phenol and chloroform were added, vortexed, and centrifuged. The upper, aqueous layer was transferred to a clear tube. Diethyl pyrocarbonate (DEPC) treated water was added to the organic layer and was again vortexed and centrifuged. The aqueous layer was combined with the first and the pooled solution was extracted with phenol and chloroform twice. The aqueous phase was further extracted with chloroform twice and was then precipitated with 0.3 M sodium acetate and two volumes of 95% ethanol. Total RNA pellet was rinsed with 70% ethanol and resuspended in DEPC treated water. To extract poly(A)<sup>+</sup>RNA from the total RNA, the sample was passed through an oligo(dT)-cellulose column (Sigma). Columns containing 0.25 ml of oligo(dT)-cellulose were regenerated by running 10 ml of sodium hydroxide through the column, then 10 to 15 ml of DEPC treated water. Finally enough column loading buffer (0.5 M sodium citrate, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS) was applied until the eluate had a pH of under 8.0. The total RNA samples were combined with an equal volume of 2x column loading buffer, heated to 65°C for 10 minutes, cooled quickly and loaded on to the column. The samples were passed through five times by reloading the eluate to the column. Unbound total RNA was removed by washing the column with 5 ml of column loading buffer. Once all the unbound RNA had been washed out, the poly(A)<sup>+</sup> RNA was eluted with elution buffer (10 mM Tris-Cl, 1 mM EDTA, 0.05% SDS). Collected fractions were quantified by placing the entire sample in a quartz cuvette that has been soaked in 1:1 concentrated HCl/methanol and washed exhaustively with DEPC-treated water. Fractions with OD<sub>260</sub> absorbing materials were pooled and kept at -80°C until needed.

#### II.8.2. End-labeling of the oligonucleotide using polynucleotide kinase

The oligonucleotide chosen for end-labeling was the same as the one used in sequencing: 5' GAG AAT TGG TTC AAT GAA TG 3'. Approximately 30 pmol of the oligonucleotide were used for end-labeling. [ $\gamma$ -<sup>32</sup>P] ATP at 5000Ci/ mmol was added

together with 8 units of polynucleotide kinase. The reaction was incubated at 37°C for one hour and then transferred to 65°C for ten minutes to inactivate the enzyme. The labeled oligonucleotide was separated from the unincorporated [ $\gamma$ -<sup>32</sup>P]ATP by gel filtration through a G-10 Sephadex column with STE (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA). Radioactivity peaks were determined by Geiger counter, with the labeled oligonucleotide appearing at the first peak eluted from the column.

### II.8.3. Primer extension of poly(A)<sup>+</sup> RNA

The poly(A)<sup>+</sup> RNA isolated above was annealed to the labeled oligonucleotide. The annealing mix had a final volume of 10  $\mu$ l and included poly(A)<sup>+</sup> RNA, labeled oligonucleotide, 10 mM PIPES at pH 6.4, and 0.4 M NaCl. The mixture was placed in a capillary tube and sealed. It was heated to 65°C in a water bath, the temperature was lowered to 41°C, which was found to be the proper annealing temperature. It remained at 41°C overnight and was then added directly to a tube containing the reactants necessary for reverse transcription. The reaction tube contained reverse transcriptase buffer (50 mM Tris-Cl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, no additional salt was added to the buffer as the hybridization mix contained NaCl), 0.5 mM of each dNTP, 2  $\mu$ g actinomycin D, and 200 units of Moloney-Murine Leukemia Virus reverse transcriptase (Promega). The annealing mix was added to the tube for a final volume of 100 $\mu$ l and it was incubated at 42°C for one hour. Yeast tRNA was added to a final concentration of 50  $\mu$ g/ml and the extension product was precipitated with 0.3 M sodium acetate and 2 volumes of 95% ethanol at -80°C. The pellet was then rinsed in 70% ethanol and resuspended in the formamide solution obtained from the T7 sequencing kit (Promega). The radiolabeled

extension product was run for 4 hours at 1000V on a 6% acrylamide gel alongside a sequencing ladder obtained from one of the deletions described above.

### III. RESULTS

#### III.1. Construction of Deletions

In order to facilitate the isolation of constructs containing a deleted insert colony hybridizations were performed on *E. coli* colonies. This method allowed the processing of 100 colonies per plate and assured that all further processing will be carried out with randomly generated 5' deletions. The patching method used limited the number of colonies that can be screened on a single plate. However, since the colonies were patched on a grid, they were bigger and easily identifiable. This reduced the possibility of picking and analyzing the wrong colonies. The autoradiogram on the following page demonstrates the difference in intensity between the DNA from colonies that hybridized to the radiolabeled probe and the DNA from those that did not. The autoradiogram was placed beneath the plate and the colonies that gave a signal (see figure 3.1) were picked and grown. Putative deletions were characterized by sequencing and ten colonies spanning the region of -449 to -74 were chosen for further analysis.

Figure 3.1.

Autoradiogram of colony hybridization obtained after Exonuclease III generation of deletions. A hundred colonies were patched onto an LB ampicillin (100 µg/ml) plate. The membranes were probed with a radiolabeled *Xba*I to *Bam*HI fragment of pDdRNR/LacZ.



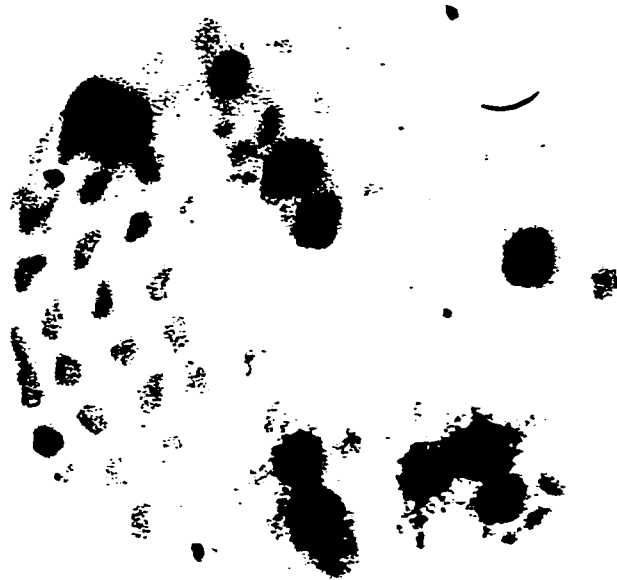


Figure 3.1. Autoradiogram of colony hybridizations.

Figure 3.2.

Sequence of the entire insert ligated to pDdGal16. Single underlining indicates the *Xho*II and *Bam*HI sites used to cut the insert out of pDdM100. These represent the two extremes of the insert. The double underlining represents the ATG site of the *mrB* gene. Italicized region located 40 bases downstream of the ATG site is the oligonucleotide binding site, used in sequencing and in primer extension.

The bold T represents the transcription start site of the *mrB* gene as determined by primer extension. It is situated at position -115 from the ATG. The putative TATA box is underlined and bold, and is located 25 to 30 base pairs upstream of the start site. The oligo (dT) run frequently seen in *Dictyostelium discoideum* promoter regions is boxed with a dashed line.

The 5' deletions analyzed in the figures above are indicated by a boldfaced base as well as the size away from the ATG site in parentheses above it. The putative regulatory boxes A-D are enclosed with a solid line.

	10	20	30	40	50	60
-1824	TTCTAAGAGT	<u>TGGATCTTCT</u>	TTAGTTAATG	AAATTTGTTG	ATTCTTTACT	AATGAAATAC
-1764	CTGCACCTCC	TGGTGGTGGT	GGTGGTGCGC	CATATTGTCC	TGCTGGTTGT	GGTGCACCAT
-1704	ATTGTCCTGC	TGGTTGTGGT	GCACCATATT	GTCCTGCTGG	TTGTGGTGCT	CCGTATTGAC
-1644	CGGCTGGTTG	TTGTTGTGGT	GGTGCTCCAT	ATTGACCGGC	TGGTTGTTGT	TGTGGTGGAT
-1584	AACCTCCTGG	TGCTCCTGGT	GGTGGTGGAG	TTGGATAACC	TCCTGGTTGT	TGTGGTGCTC
-1524	CAGTTGGTGG	TCTTTGTTGT	TGTTGTGGTG	GATAGCCTCC	TGGTGTGTTGT	GTAGTATTAG
-1464	ATGGTTGTGG	TGGTAAAAAT	TGATTAGCTT	GTGGGTTTGG	GTTTGATACA	CCCGGTGTGG
-1404	ATGATGGTTG	TCTATAATAA	TTATTATTAC	CTTGTGAACC	AGATGGTGGT	GGTGGGTTAT
	<i>capA</i> ←					
-1344	ACATTTTTAT	TTTTTATATA	TATAAATTAT	ATATTCTATT	AGAAAAAAAA	AAAAAAAAAGA
-1284	AAAAAGAAAA	AAAAAAAAAA	AAAAAATGAA	AATTAATAAT	TTGAAAATTA	AAAAAAAAAA
			(-1200)			
-1224	AAAAAATAAA	AGAAAAAATA	AATAAAAAAT	AAAAAATAAT	AATTTAAAAT	AAATAAAATT
-1164	TTAAATTAAT	AAAAAAAAAA	ATAAAAATGG	TGAAAATTTT	GAAAGTGATA	ATATTAGATA
-1104	TTTATTGTTT	AATATTTACA	CTGTTGTATG	TGGAATAGTT	TTTTTATTTT	TTAGAATTTT
-1044	TTTTTTTTTT	TCTTATTTTT	TTTTTTATTG	TTTTTAATTT	TTTTTTTTTT	TTTCAATAA
			(-961)			
-984	AATTTTTTGC	TTGATATCTC	ACAAAAGTCT	AAAATTCTAA	GGTCACCCTC	ACAAAAAAT
-924	GATCAATGGC	TGTTTAATTA	TTAATGCCAC	CAATACGGAG	TGAATTATTA	TTATAATATA
			(-846)			
-864	TTCATTGAAT	AATTTTTTTT	TTTTTTTTTT	TTTTTTATTT	TTTTTATTTT	TTTTATTTTT
				(-772)		
-804	TTTTTTTATT	TTAATTTATT	TGAAAATTTT	AATACAAAAC	AAATAAAATT	ATTTTTAAAA
-744	AAAACCTGGT	AAATGATCAT	TTATTTATTT	TTTTTTTTAA	ATTTTATTTT	TTGTCTAACA
-684	CCAGAGTCTG	AAAATTTCAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAGA
				(-588)		
-624	TTTTTTAAAC	CAACAAAAAA	TCAATTTTCC	TTAAATTTAA	TTAAAGAGTT	ATTTATTGCA
-564	CTGAAATGAT	TTATTGTAAT	TGTATAATTT	TTTTTTTTTT	TTTATTTTTT	TTTGAATTAT
						(-449)
-504	ATGATTTATT	AATATCACCA	ACTTTTTGTA	AAATAATAAA	TTATATTTTT	TTTTTCTAGA
	BOX D	(-430)	<i>KpnI</i>			<i>XbaI</i>
-444	<u>ATCGGAGTGG</u>	<u>TACCCA</u> AATA	TAGCTTTTTT	TTTTTTTTTT	GAAAAATTAT	TAAAATTTAA
		(-370)	BOX C	(-345)		
-384	TTTATT <u>GGAA</u>	<u>CGGTGATTCC</u>	<u>ATCATAACA</u>	AAAACAAATA	AAATAAATAA	AATTTTAATA
		(-310)	(-305)	(-295)	(-281)	
-324	TTTTTTTCTT	<u>TCCGGAATGT</u>	<u>GTGTGTATTT</u>	TCCAAAAAAA	AAAAAATAT	TCTGTTTTTT
	BOX B			(-223)	(-213)	
-264	TTAAAAAATA	GAAATTAATT	TTTTAT <u>AGGA</u>	<u>ACCAAAATG</u>	<u>CGCT</u> AAAAAT	TTAAAAAATA
			BOX A			<i>DraI</i>

	10	20	30	40	50	60
	----- ----- ----- ----- -----					
-204	ATAAAAAATT	CAAATTTTTT	TTTTTTTTTT	TTTACTTTT	TTTTTTTTTT	TATTATTATT
	(-136)	(-131)		→ transcriptional start site		
-144	<u>TATAACAAAT</u>	AAATATTATT	TTTTAATTTT	ATTATTCATA	TTATATAATT	TTGGATTGAT
	(-74)					
-84	TTCAAAACTT	AATAAAATCT	CATTGTACAT	TAAATTTTAT	AAAATAAAAT	AAAAAAAAAA
			+1			
-24	AAAATTAAAA	AATAAAAAAT	AAAAATGGAA	GAAATTAATA	AAAAGATAC	ATTCATTGAA
			→ <i>rnrB</i>			
37	CCAATTCTCA	AAGAAAATAA	AGATAGATTT	GTATTATTTC	CAATAAAATA	CCCAGATATC
97	TGGAGAATGT	ATAAAAAAGC	CCTTGCAAGT	CATTGGGTAG	CTGAAGAAAT	TGATCTTGGT
157	AAATTATCTA	TACATATTTT	ATTATTATTA	TTATTATTAT	TATTATTATT	ATTATTATTA
217	TTATTATTAT	TATTATTATT	ATTATTATTA	TTATTATTAT	TATTATTATT	GTATATATTA
277	ATTTATTAAT	TAATTTTATT	AATTTTATTA	TTAGGTAATG	ATAATGTTGA	TTGGGAATAT
337	AAATTAACAG	ATAATGAACG	TCATTTTCATC	TCACATGTTT	TGGCATTTTT	TGCTGCATCT
397	GATGGTATTG	TAAATGAAAA	CCTTGCAACT	AGATTTATGT	CTGAAGTACA	AATTCAGAA
457	GCAAGATGTT	TCTATGGTTT	TCAAATTGCA	ATTGAAAATA	TACATTCTGA	AACTTATAGG
517	TAAATATTTA	TCATATATAT	ATATAAAGTT	TTAATAAAGA	AAAAGTAGAT	TCTAATATAT
577	AATTTTTTTT	TTTTTTTTTT	TTTTTTTTTT	TTTTTTTTTT	TTTTTTTTTT	TTTTTTAGTC
637	TTTTAATTGA	AACTTATATT	AAAGATAAGC	AAACTAAAGA	TAAATTATTT	AATGCAATTG
697	AAACAATTCC	ATGTATTAAA	AAGAAGGCAG	AATGGGCATT	AAGATGGATA	AATGATTCAG
757	ATTCATTTGC	AGAACGTTTG	GTTGCATTTG	CAGCAGTTGA	GGGTATATTT	TTCAGTGGAA
817	GTTTTTGTTT	AATATTTTGG	TTGAAAAGA	GAGGTTAAT	GCAAGGTTTA	ACATTTTCAA
877	ATGAATTAAT	TTCACGTGAC	GAAGGTTTAC	ATTGTGATTT	CGCATGTCTT	CTCTACACAA
		<i>Bam</i> HI				
937	AGTTACAAAG	AAAGTTGGAT	CCAAAAGTCA			

Figure 3.2. Sequence of 2.7 kb insert cloned into pDdGal16.

### III.2. ONPG Assays of Various Deletions

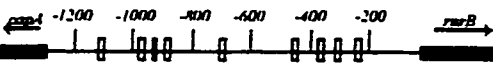
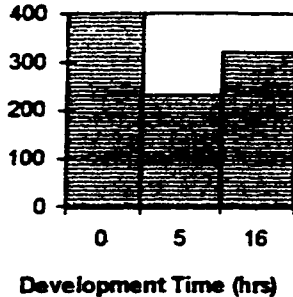
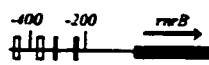
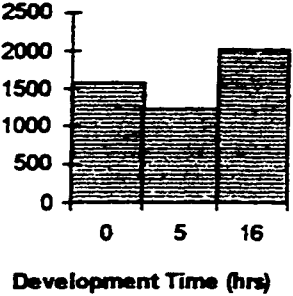
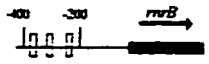
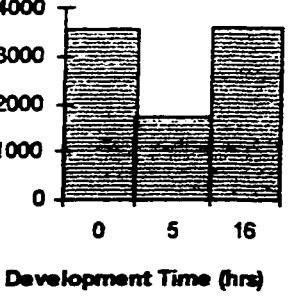
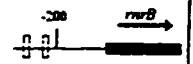
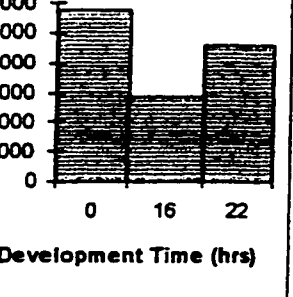
To monitor expression levels *Dictyostelium discoideum* cells were transformed with derivatives of pDdRNR/LacZ plasmid with deletions of intergenic sequences (this study) as well as internal deletions prepared by Claire Bonfils (unpublished data). In each table the deletion size given is the distance away from the ATG of the ribonucleotide reductase gene. The maps included show the size of the deletions and the G/C rich boxes (represented by vertical rectangles) remaining.

*Dictyostelium discoideum* cells were harvested at three time points- zero hour indicating vegetative cells, 5 hour; early aggregation, and 16 hour; slug.  $\beta$ -galactosidase activity of the harvested cells was then assayed. Vegetative level of activity indicates protein activity at zero hour while resurgence of activity involves an increase of activity of the  $\beta$ -galactosidase during late development at the slug stage. Vegetative and resurgent activities were determined to be positive if they were higher than the activity measured at the 5 hour time point, and negative if they were lower or equivalent to it. If the activity was only negligibly higher it was termed 'low'. If specific activity was found to be lower than 50, levels were considered negative.

The 5' deletion results indicate that the deletions upstream of -345 show a profile similar to that of the full length insert. Downstream of -345 the activity no longer exhibits developmental activity while downstream of -213 vegetative activity is lost as well.

The internal deletions indicate that developmental activity is lost when two of the three G/C rich boxes between -430 and -213 are deleted, while the G/C rich box spanning -238 to -221 reduced vegetative activity but did not eliminate it. None of the internal deletions analyzed completely abolished vegetative activity while retaining developmental activity.

Table 3.1.  $\beta$ -galactosidase Assays of Deletions between *Xba*I and *Dra*I

Deletion or Construct	$\beta$ -Galactosidase enzymatic activity	Vegetative levels	Resurgence								
<p>RNR</p> 	<p>Specific Activity</p>  <table border="1"> <caption>Specific Activity Data for RNR</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~400</td> </tr> <tr> <td>5</td> <td>~250</td> </tr> <tr> <td>16</td> <td>~350</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~400	5	~250	16	~350	+ive	+ive
Development Time (hrs)	Specific Activity										
0	~400										
5	~250										
16	~350										
<p>-449 (<i>Xba</i>I)</p> 	<p>Specific Activity</p>  <table border="1"> <caption>Specific Activity Data for -449</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~1500</td> </tr> <tr> <td>5</td> <td>~1200</td> </tr> <tr> <td>16</td> <td>~2000</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~1500	5	~1200	16	~2000	low	+ive
Development Time (hrs)	Specific Activity										
0	~1500										
5	~1200										
16	~2000										
<p>-430 (<i>Kpn</i>I)</p> 	<p>Specific Activity</p>  <table border="1"> <caption>Specific Activity Data for -430</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~3500</td> </tr> <tr> <td>5</td> <td>~1500</td> </tr> <tr> <td>16</td> <td>~3500</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~3500	5	~1500	16	~3500	+ive	+ive
Development Time (hrs)	Specific Activity										
0	~3500										
5	~1500										
16	~3500										
<p>-345</p> 	<p>Specific Activity</p>  <table border="1"> <caption>Specific Activity Data for -345</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~5500</td> </tr> <tr> <td>16</td> <td>~2500</td> </tr> <tr> <td>22</td> <td>~4500</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~5500	16	~2500	22	~4500	+ive	+ive
Development Time (hrs)	Specific Activity										
0	~5500										
16	~2500										
22	~4500										

$\beta$ -galactosidase Assays of Deletions between *Xba*I and *Dra*I (continued)

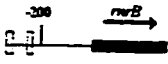
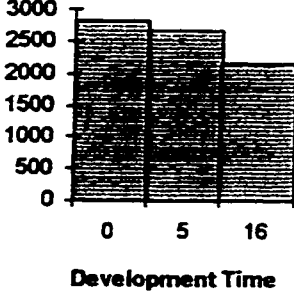
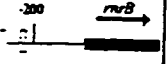
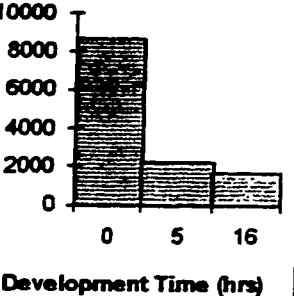
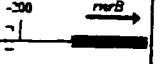
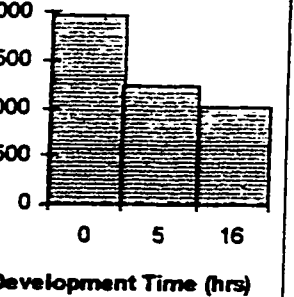
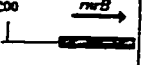
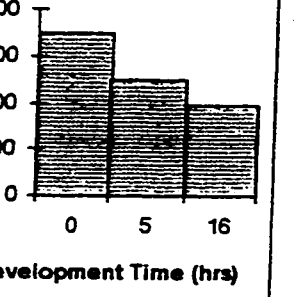
Deletion or Construct	$\beta$ -Galactosidase enzymatic activity	Vegetative levels	Resurgence								
<p>-305</p> 	<p>Specific Activity</p>  <table border="1"> <caption>Specific Activity for -305</caption> <thead> <tr> <th>Development Time</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~2800</td> </tr> <tr> <td>5</td> <td>~2500</td> </tr> <tr> <td>16</td> <td>~2000</td> </tr> </tbody> </table>	Development Time	Specific Activity	0	~2800	5	~2500	16	~2000	+ive	-ive
Development Time	Specific Activity										
0	~2800										
5	~2500										
16	~2000										
<p>-295</p> 	<p>Specific Activity</p>  <table border="1"> <caption>Specific Activity for -295</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~8500</td> </tr> <tr> <td>5</td> <td>~2000</td> </tr> <tr> <td>16</td> <td>~1500</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~8500	5	~2000	16	~1500	+ive	-ive
Development Time (hrs)	Specific Activity										
0	~8500										
5	~2000										
16	~1500										
<p>-281</p> 	<p>Specific Activity</p>  <table border="1"> <caption>Specific Activity for -281</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~1900</td> </tr> <tr> <td>5</td> <td>~1200</td> </tr> <tr> <td>16</td> <td>~900</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~1900	5	~1200	16	~900	+ive	-ive
Development Time (hrs)	Specific Activity										
0	~1900										
5	~1200										
16	~900										
<p>-213 (<i>Dra</i>I)</p> 	<p>Specific Activity</p>  <table border="1"> <caption>Specific Activity for -213 (<i>Dra</i>I)</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~3500</td> </tr> <tr> <td>5</td> <td>~2400</td> </tr> <tr> <td>16</td> <td>~1800</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~3500	5	~2400	16	~1800	+ive	-ive
Development Time (hrs)	Specific Activity										
0	~3500										
5	~2400										
16	~1800										

Table 3.2.  $\beta$ -galactosidase Assays of Deletions Downstream of *DraI*

Deletion	$\beta$ -Galactosidase enzymatic activity	Vegetative levels	Resurgence
-136	<p>Specific Activity</p> <p>Development Time (hrs)</p>	-ive	-ive
-131	<p>Specific Activity</p> <p>Development Time (hrs)</p>	-ive	-ive
-74	<p>Specific Activity</p> <p>Development Time (hrs)</p>	low	-ive
Vector Only	<p>Specific Activity</p> <p>Development Time (hrs)</p>	-ive	-ive



Table 3.3.  $\beta$ -galactosidase Assays of Internal Deletions

Deletion	Deletion map	$\beta$ -Galactosidase enzymatic activity	Vegetative levels	Resurgence								
i(430-381)		<p>Specific Activity</p> <table border="1"> <caption>Specific Activity Data for i(430-381)</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~2200</td> </tr> <tr> <td>5</td> <td>~1200</td> </tr> <tr> <td>16</td> <td>~1800</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~2200	5	~1200	16	~1800	+ive	+ive
Development Time (hrs)	Specific Activity											
0	~2200											
5	~1200											
16	~1800											
i(430-280)		<p>Specific Activity</p> <table border="1"> <caption>Specific Activity Data for i(430-280)</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~6000</td> </tr> <tr> <td>5</td> <td>~3500</td> </tr> <tr> <td>16</td> <td>~4800</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~6000	5	~3500	16	~4800	+ive	+ive
Development Time (hrs)	Specific Activity											
0	~6000											
5	~3500											
16	~4800											
i(430-213)		<p>Specific Activity</p> <table border="1"> <caption>Specific Activity Data for i(430-213)</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~1800</td> </tr> <tr> <td>5</td> <td>~1200</td> </tr> <tr> <td>16</td> <td>~1000</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~1800	5	~1200	16	~1000	+ive	-ive
Development Time (hrs)	Specific Activity											
0	~1800											
5	~1200											
16	~1000											
i(370-310)		<p>Specific Activity</p> <table border="1"> <caption>Specific Activity Data for i(370-310)</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~1700</td> </tr> <tr> <td>5</td> <td>~900</td> </tr> <tr> <td>16</td> <td>~1500</td> </tr> </tbody> </table>	Development Time (hrs)	Specific Activity	0	~1700	5	~900	16	~1500	+ive	+ive
Development Time (hrs)	Specific Activity											
0	~1700											
5	~900											
16	~1500											

$\beta$ -galactosidase Assays of Internal Deletions (continued)

Deletion	Deletion map	$\beta$ -Galactosidase enzymatic activity	Vegetative levels	Resurgence								
i(370-280)		<p>Specific Activity</p> <table border="1"> <caption>Specific Activity Data for i(370-280)</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~5500</td> </tr> <tr> <td>5</td> <td>~2500</td> </tr> <tr> <td>16</td> <td>~4200</td> </tr> </tbody> </table> <p>Development Time (hrs)</p>	Development Time (hrs)	Specific Activity	0	~5500	5	~2500	16	~4200	+ive	+ive
Development Time (hrs)	Specific Activity											
0	~5500											
5	~2500											
16	~4200											
i(370-213)		<p>Specific Activity</p> <table border="1"> <caption>Specific Activity Data for i(370-213)</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~1500</td> </tr> <tr> <td>5</td> <td>~700</td> </tr> <tr> <td>16</td> <td>~900</td> </tr> </tbody> </table> <p>Development Time (hrs)</p>	Development Time (hrs)	Specific Activity	0	~1500	5	~700	16	~900	+ive	low
Development Time (hrs)	Specific Activity											
0	~1500											
5	~700											
16	~900											
i(310-213)		<p>Specific Activity</p> <table border="1"> <caption>Specific Activity Data for i(310-213)</caption> <thead> <tr> <th>Development Time (hrs)</th> <th>Specific Activity</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>~1200</td> </tr> <tr> <td>5</td> <td>~1200</td> </tr> <tr> <td>16</td> <td>~2800</td> </tr> </tbody> </table> <p>Development Time (hrs)</p>	Development Time (hrs)	Specific Activity	0	~1200	5	~1200	16	~2800	-ive	+ive
Development Time (hrs)	Specific Activity											
0	~1200											
5	~1200											
16	~2800											

### III.3. Primer Extension

Primer extension was carried out to determine the transcriptional start site of the *mrB* gene. The primer used was the same one used for the sequencing performed on the deletions. A sequencing ladder of deletion -213 was run alongside the primer extension product. The transcriptional start site of the *mrB* gene was found to be at site -115 from the first codon of the *mrB* gene.

Figure 3.3.

The primer extension reaction was run next to a sequencing ladder of one of the deletions (-213) and its location is indicated by an arrow. The base corresponding to the primer extension product is boldfaced.

Reverse transcription was carried out using M-MLV reverse transcriptase with ~5  $\mu\text{g}$  *Dictyostelium discoideum* poly(A)<sup>+</sup> RNA. Poly(A)<sup>+</sup> RNA extraction as well as primer extension conditions are described in Materials and Methods.

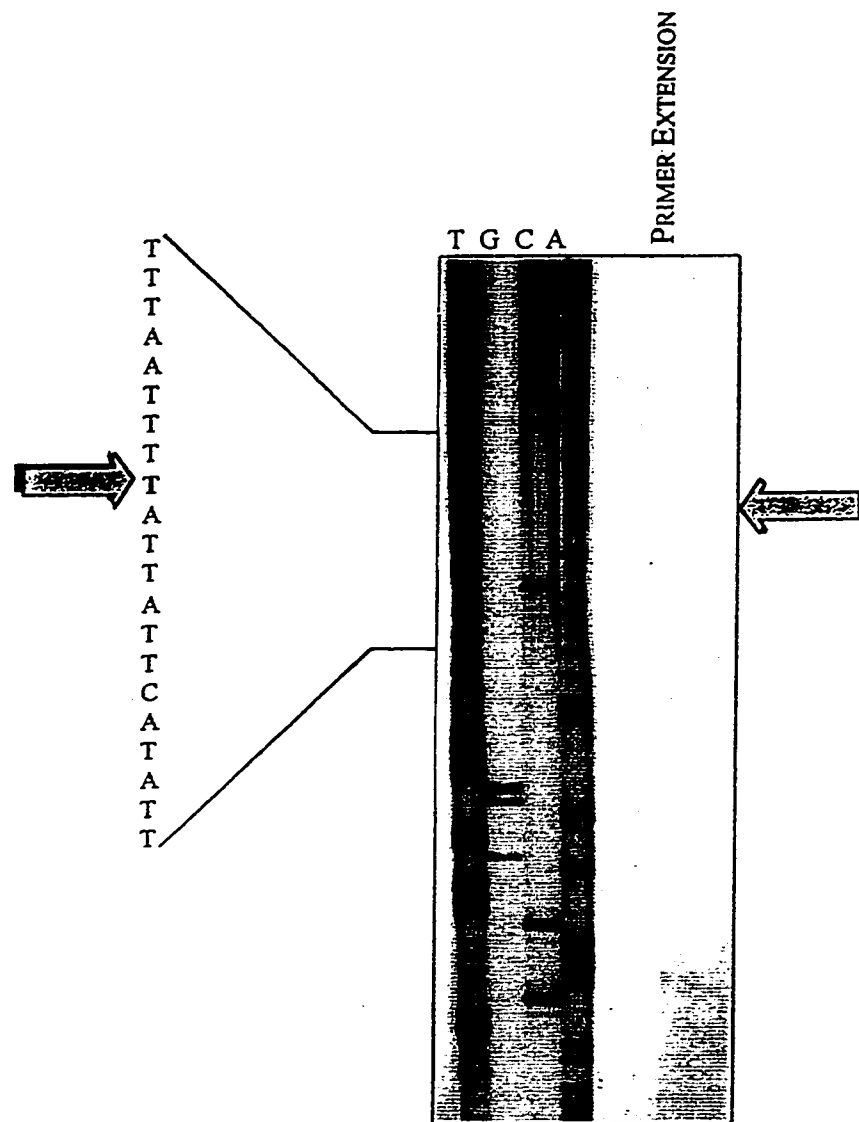


Figure 3.2. Primer extension analysis of *rnrB* gene.

## IV. DISCUSSION

This work deals with isolating and analyzing the promoter regions upstream of the gene, *mrB*, which encodes the small subunit of ribonucleotide reductase. Prior to the start of this project, DNA synthesis was known to fluctuate during development. Synthesis would be initially high at the onset of development and would drop within a few hours. A resurgence in synthesis would then be observed during late development at the slug stage [Zada-Hames and Ashworth, 1978]. The site of DNA synthesis was localized to prespore cells [Zimmerman and Weijer, 1993].

The gene encoding the small subunit of ribonucleotide reductase was first isolated in Dr. Tsang's lab. A Northern blot analysis was carried out, which indicated the levels of *mrB* transcript fluctuated during development. The fluctuation in transcript levels corresponded well with the changes in the rate of DNA synthesis during development.

Initially, pDdRNR/LacZ, a plasmid with the noncoding region and a segment of the coding region fused to a LacZ reporter gene (see Figure 2.1), was constructed. The cells transformed with the pDdRNR/LacZ construct showed a  $\beta$ -galactosidase activity profile very similar to the *mrB* mRNA profile. As well, histochemical staining showed that the  $\beta$ -galactosidase activity was localized exclusively in prespore cells, providing a spatial correlation between *mrB* expression and DNA synthesis [Tsang et al., 1996]. These results indicated that the reporter gene system adequately represented the endogenous *mrB* gene activity and could be used to further delineate the *cis*-elements involved in regulating *mrB* expression during development.

The noncoding sequence flanked by the *capA* gene and the *mrB* gene is 1340 bp long. Since the construct generated appeared to have a  $\beta$ -galactosidase activity profile and an RNA profile (see figure 3.1) similar to that observed for the endogenous RNA levels it

would be reasonable to assume that the ribonucleotide reductase regulatory sites were located somewhere in this large span of sequence. In order to identify these elements, 5' deletions were generated using the pDdRNR/LacZ construct.

Initially, *XbaI* and *KpnI* were used to generate two smaller constructs. Each of these was present once in the vector, in the multiple cloning site, and once in the insert, in the middle of the noncoding area. These two deletions were important for two reasons. First, if their levels of  $\beta$ -galactosidase during development proved to be the same as for the parent construct, half of the noncoding sequence could be eliminated. As well, the span between the two sites is short and GC rich, pointing to a potential regulatory domain. Cells transformed with these two constructs showed that both deletions had the same  $\beta$ -galactosidase profile as the original pDdRNR/LacZ, indicating that the promoter regions were likely to be downstream of *KpnI* and that this particular GC rich sequence might not constitute a site involved in the temporal regulation of *mrB*.

Another important deletion that was generated by using restriction enzymes is pDdDra/LacZ. The more distal *DraI* site in the noncoding region is located 213 bp upstream from the ATG. The construct is important for two reasons. First, the deletion is located downstream of the *KpnI* cut site, so it is an interesting construct to analyze. Also, it was generated using a restriction enzyme rather than by digesting the ends of the DNA through the use of exonucleases. This means that the exact size of the construct is known and the exact site of deletion can be pinpointed on the sequence. This deletion, as well as the pDdXba/LacZ or pDdKpn/lacZ, were therefore used as markers on agarose gels, to estimate the approximate sizes of the randomly generated deletions.

Other means had to be used to generate the bulk of the deletions as there were very few other convenient cut sites between the *KpnI* site at -436 from the translational start site and the ATG. Three common techniques were used to generate all the 5' deletions required for a thorough analysis of the region. The three protocols involved the use of pancreatic

DNaseI, ExonucleaseIII, and Bal31 nuclease, respectively. I was responsible for the DNaseI and the ExonucleaseIII experiments while another student in the lab, Zeina Saikali, carried out the Bal31 nuclease protocol.

DNaseI deletions yielded a very small number of deletions compared to the overall number of colonies screened. When sized by agarose gel electrophoresis, most proved to be about the same size. Ultimately, only three deletions isolated in this manner were used. The reason for the low numbers of useful deletions is not known, but might be due to DNaseI hotspots in the insert sequence.

ExonucleaseIII and Bal31 nuclease are both exonucleases digesting the ends of linear DNA. The difference between the two is their rate of digestion; while ExonucleaseIII ranges from 80 bp/min. at 22°C to 600 bp/min. at 45°C, Bal31 is much slower and digests the DNA at about 19 bp/min. in a 100 µl reaction containing 10 µg of DNA and 0.5 unit of enzyme [Sambrooke et al., 1989]. ExonucleaseIII was therefore used to digest pDdRNR/LacZ in order to generate deletions upstream of the *Xba*I cut site, while Bal31 was used on pDdKpn/LacZ to generate deletions closer to the ATG site. The deletions upstream of *Xba*I were made in order to confirm the profile found for pDdXba/LacZ.

By sizing all the deletions in this way, only the ones that were found to be of the appropriate size, namely downstream to or near the *Kpn*I cut site, were then sequenced.

Sequencing the deletions was facilitated by the fact that the sequence for the insert was known. The purpose was therefore merely to pinpoint the exact site of the deletion. This was accomplished for many constructs downstream of the *Kpn*I site. A couple of deletions located upstream of *Xba*I were sized by end-labeling a fragment of the insert and running it alongside a sequencing reaction which was used as ladder. While this provided an approximate sizing, the results were not optimal, and this technique was not repeated. The remaining deletions upstream of *Xba*I were sized by agarose gel electrophoresis.



Three time points during the developmental cycle of *Dictyostelium discoideum* were chosen for analysis. A vegetative time point referred to as zero hour, where the cells were removed from the nutrients and frozen immediately. A 5 hour time point, prior to aggregation, and a later time point at 16 hours during slug development were selected. These points were chosen as they are representative of the profile observed for the parent construct; an initial high  $\beta$ -galactosidase activity, then a drop by five hours, followed by a resurgence of enzyme activity levels during late development.

The deletions upstream of the *XbaI* site had a profile identical or very similar to the one observed for the full length insert. The overall trend suggests that this broad region does not play an important role in the control of *rnrB* expression.

The second region analyzed, the sequence between the *XbaI* site and the *DraI* site proved to be the critical region controlling gene expression. The trend described above was observed for all deletions up to -345. Beyond this point, the vegetative  $\beta$ -galactosidase levels were high and dipped during early development but did not go back up during later development. This new trend was then repeated in three more deletions spanning the region of -305 to -213 (the *DraI* site). Clearly, a regulatory domain was isolated between -345 and -305 upstream of the ATG.

Another important controlling element was observed downstream of the *DraI* site. When the insert is deleted to 136 bases of the ATG start site, the specific activity observed drops to background levels. Other deletions, even closer to the ATG, show the same low levels. Vegetative activity of the enzyme is knocked out of deletions downstream of -213. The data obtained from the 5' deletion analysis of the promoter region seem to indicate that the two activities observed during the developmental cycle in *Dictyostelium discoideum* are attributable to separate promoter regions; with vegetative levels controlled downstream of -213 and developmental activity localized to the region spanning -345 to -305.

5' deletions alone cannot determine complex promoter structures as the method eliminates a large span of sequence. This can cause disruption of DNA structure and might affect interactions with *trans*-acting factors. As well, there may be regulatory domains located in different areas of the sequence that might function together to control transcription. Such interactions cannot be observed by 5' deletions. Internal deletions can be constructed to delete a specific region or regions. This is a more accurate analysis as the remainder of the insert is left untouched.

The element that seemed to control vegetative  $\beta$ -galactosidase activity levels is an A/T rich region located between -213 and -136. There are other *Dictyostelium discoideum* genes known to be regulated in this way. A/T rich domains control the activity of two actin genes, *actin 6* and *actin 15*. Both transcripts have two A/T rich boxes regulating gene expression as well as an oligo(dT) box that has been shown to affect level of expression of *actin 15* [Hori and Firtel, 1994].

Noncoding regions of *Dictyostelium* DNA have very low G/C content [Pavlovic et al., 1989], and therefore all G/C rich zones in the *Xba*I to *Dra*I region may represent regulatory elements. Four such areas were found and labeled A to D boxes. Box A, AGGAACCAAAATTGCGCT, is closest to the ATG, spans the sequence from -238 to -221, and is 47% C/G. Box B, CGGGAATGTGTGTGTGT, is between -313 and -299 and has a G/C content of 57%. The next G/C box (box C), GGAACGGTGATTCCA, is also composed of 57% G/C and is found between -378 and -364. Finally box D, CTAGAATCGGAGTGG TACCCA, situated -449 to -429 from the ATG, encompasses the *Xba*I and *Kpn*I cut sites, and is 55% G/C. The 5' deletions analyzed indicate that either the GC rich pocket spanning -313 to -305 of B is responsible for the loss of resurgent  $\beta$ -galactosidase activity, or that the loss of activity might be due to the AT rich domain located upstream of box B. Other elements might contribute to developmental gene regulation as well.

The four G/C boxes were analyzed by selectively removing one or more of them at a time while keeping the upstream sequence intact. Removal of box A resulted in a reduction of vegetative activity. This is not expected since removal of box A did not eliminate or reduce vegetative expression in the 5' deletions. One explanation is that box A is the one closest to the A/T rich domain located between -213 to -136. Deletion -213 in the 5' deletion series joined the A/T rich domain to the multiple cloning site upstream of the *Bgl*III cut site [CCAGAATTCTAAGCTTATA] in the pDdGal16 vector. This region has a G/C rich area similar in composition to box A and might have therefore substituted for it. The internal deletion missing box A, on the other hand, eliminates the G/C rich box but leaves the rest of the sequence upstream of box A untouched. This result indicates that the vegetative activity is due to the A/T rich domain downstream of -213, and that box A plays a role in regulating the level of expression. From the RNA data, box A clearly plays a role in late regulation [Bonfils, unpublished data].

Results of the internal deletions indicate that removal of the B or the C box does not affect the activity profile at all; vegetative and developmental activities were all akin to wild type.

The removal of two elements at the same time also yielded some interesting results. Removal of boxes B and C did not affect the activity of the  $\beta$ -galactosidase. A reduced developmental resurgence is observed for the A and B internal deletion. From the RNA data, boxes A and B are involved in repression of the developmental activity. Moreover, a low vegetative activity is predicted, since the A box is not present. The specific activity obtained for the vegetative sample is very low compared with other internal deletions.

Internal deletions were constructed that were missing the -213 to -136 domain together with the G/C rich boxes. These internal deletions showed only background activity (results not shown). These results can be due to the lack of a vegetative promoter box, and the disruption of the TATA box.

The high variability in the specific activity values obtained are probably due to differences in transcript copy number in the various transformations. An example of this variability are the activity profiles of deletions -449, -430, and -345. While the profile for each is very similar, the specific activity values at the vegetative time point rise from 1566 pmol/sec/mg (-449) to 3547 pmol/sec/mg (-430) and then go back down to 2578 pmol/sec/mg (-345). Due to this fluctuation, the data was analyzed by comparing the fluctuations observed during development between deletions rather than by comparing absolute specific activity values. Southern blot analyses would have to be carried out in order to normalize the data obtained, and to determine the effect that copy number has on the specific activity values.

The situation observed is more complex than anticipated, with different promoter blocks interacting at different times during development. Results indicate that box B is required for developmental activity but is only functional with box A, a second G/C box. The vegetative activity levels might depend upon box A working alone or with an A/T rich domain located near the transcriptional start site. The regulating element within that region might include the oligo(dT) box spanning the sequence from -190 to -150.

Ribonucleotide reductase is not the only protein to be regulated in this way. The *Dp87* gene [Morio et al., 1994] promoter is divided into seven distinct elements with positive and negative regulating elements functioning together to regulate the sorus matrix protein throughout development. Other examples of promoter elements interacting during development include the *ras* gene promoters, in which a CA repeat (or CAE: CA element) works in association with a negative regulatory element, as well as the *SP60* and *SP70* gene elements [Haberstroh et al., 1991; Fosnaugh and Loomis, 1993]. The two first CA elements in *SP60* gene promoter region function synergistically, and removal of one or the other does not affect protein expression to the same extent as deletion of both elements. Deletions carried out in the 5' and 3' region of the *SP70* gene promoter indicated that, while levels of protein expression decrease, the pattern of expression as well as cell-type

specific expression remains the same. From this it was concluded that various elements in the noncoding sequence “must be closely opposed or even interdigitated” [Fosnaugh and Loomis, 1993].

Further evidence of the model presented is that the removal of boxes A, B, and C eliminated gene expression during late development. The lack of resurgence is due to the elimination of the three C/G rich boxes. The vegetative expression is at normal levels since the A/T rich region downstream of box A was not removed.

The transcriptional start site of the gene in developing cells was determined by primer extension. The data suggests that in developing cells there is a single start site 115 bases upstream of the ATG. This is consistent with profiles obtained of deletions missing this area (such as -74), which were found to be no higher than background levels. As well, it would explain the lack of activity observed for the two deletions upstream of the start site, -131 and -136. While these still possess the start site, they are both deleted downstream of the putative TATA box, situated from -139 to -144. However, mRNA from vegetative cells was not analyzed and other transcriptional start sites might exist in these cells.

The noncoding region present in the pDdRNR/LacZ construct has been shown to contain the promoter elements required by the *mrB* gene. The region flanked by *capA* and *mrB* is large and in order to determine the critical sites, 5' deletions as well as internal deletions were constructed and assayed. These were used to pinpoint the promoter sites present in the construct. The primer extension data was carried out to provide insight into the start of transcription and length of the mRNA transcript. It has also indicated the largest feasible deletion that can be constructed as anything deleted beyond the start site does not have any activity.

The information obtained from these analyses can be used to manipulate the endogenous gene and track the fate of the affected cells. The vegetative activity and the late developmental activity of RNR seem to be controlled by different combinations of promoter

elements. The vegetative activity levels are controlled by an A/T rich domain closer to the transcriptional start site, while the elevated levels observed during late development seem to be controlled by box B working in conjunction with a second G/C rich element adjacent to it. It should therefore be possible to specifically knock out the developmental expression of the mRNA and determine the effect, if any, that such a mutation would have on the developmental cycle of *Dictyostelium discoideum*.

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## APPENDIX

Calculated  $\beta$ -galactosidase specific activities.

Deletion Name	Vegetative (0 hr)	Range	5 hr.	Range	16 hr.	Range
RNR	400	31	227	39	327	16
-449( <i>Xba</i> I)*	1566		1211		2011	
-430( <i>Kpn</i> I)	3547	766	1730	672	3600	548
-345	2578	417	1641	454	2666	175
-305	2797	143	2642	0	2156	102
-295	8649	710	2174	147	1635	15
-281	1953	73	1211	16	1007	18
-213( <i>Dra</i> I)*	3480		2470		1924	
-136	40	15	13	3	12	1
-131	16	0	13	1	23	4
-74	100	9	50	1	--	
Vector only*	7		15		3	
i(430-381)	2188	51	1321	0	1848	134
i(430-280)	6064	295	3579	17	4918	279
i(430-213)	1929	11	1287	140	1056	78
i(370-310)	1799	90	922	0	1579	206
i(370-280)	5636	549	2647	10	4269	471
i(370-213)	1568	208	761	77	956	444
I(310-213)	1218	0	1252	514	2892	338

note: Duplicate data was not available for the samples labeled with a star.