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Molecular Studies On The Induction Of Amylolytic
Expression In The Yeast Schwanniomyces castellii

by

© Terrance Michael Dowhanick B.Sc.

A thesis submitted to the Faculty of Graduate Studies in
partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Biology Department
Carleton University
Ottawa, Ontario
March, 1988

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ISBN 0-315-40603-8

The undersigned hereby recommend to the Faculty of Graduate Studies acceptance of this thesis, submitted by Terrance Michael Dowhanick, B.Sc., in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The budding yeast Schwanniomyces castellii secretes the starch-degrading enzymes α -amylase and glucoamylase. In previous work, these enzymes were shown to be differentially expressed, although molecular details of these events were never studied. Using both enzymatic assays and a polyclonal anti-glucoamylase antibody as a probe for glucoamylase, molecular studies at the level of transcription and translation were carried out to determine the expression and repression of Schwann. castellii glucoamylase. These studies established that glucoamylase expression is induced by starch and maltose and repressed by glucose. Both induction and repression of glucoamylase are regulated at the level of transcription, taking place within 30 minutes after exposure to the respective sugars. Sugars such as cellobiose, galactose, lactose, and xylose or no sugar result in a low level of expression. Glucoamylase transcription is also repressed by the glucose analog 2-deoxyglucose, and by heat shock, and is not derepressed by cyclic AMP. The glucoamylase undergoes at least two post-translational modifications: the 110-120 kDa nascent, preglucoamylase is converted to a 138 kDa cell-associated precursor, and then to an active, extracellular 146 kDa protein.

Poly A⁺ - RNA isolated from maltose-induced cells was converted into a cDNA library using M13 mp19 and selectively

screened by cascade and 'differential' hybridization. Two clones (Cas-2,3A) specific to maltose induction were found. These related clones coded for a single transcript about 21s (3.15-3.25 kilobases) that could code for a protein similar in size to the preglucoamylase. Cas-3A cDNA was used to identify the corresponding genomic sequence, which was located within a 7.6 kbp Eco RI fragment. This fragment was cloned into λ Charon 4A and designated Scas-1. The 21s transcript was located within a 3.8 kbp segment of Scas-1 mapped by restriction endonucleases and nucleotide sequencing. The expression pattern of Scas-1 was shown to be similar to that of the nascent 120 kDa glucoamylase translated in vitro. This gene is transcribed in Schizosaccharomyces pombe with the regulatory sequences appearing to be partially recognized, although the appearance of RNA components and no detection of product translated in vitro implies that incomplete splicing of two putative introns might be occurring or that the RNA is not intact.

I wish to dedicate this work to my parents, Walter and Phyllis, for their love and support which were especially important to me in my formative years, and to my wife Kimberly, for her love, support, and unlimited patience in my Graduate years.

ACKNOWLEDGEMENTS

My special thanks to Dr. Vern Seligy for his guidance, encouragement, constructive criticism, and tireless dedication to teaching, which were all instrumental to the completion of this project.

Special thanks are extended to Dr. Graham Stewart and Mrs. Inge Russell of the Production Research Department of Labatt Brewing Company Limited for their encouragement and financial assistance over the years.

Thanks are extended to the National Research Council of Canada for permitting me to conduct my research in their labs as part of a PILP contract agreement between them and Labatts. I am indebted to Mr. Michael Dove for his skill and patience in teaching me numerous techniques and for the major role played in the sequencing and computer analysis of the Scas-1 gene. I thank Dr. Gordon Willick for purification of the crude glucoamylase and preparation of the anti-glucoamylase sera, Dr. Wing Sung for preparation of the synthetic oligonucleotide primers, and to Mr. Harry Turner for photographic assistance. I wish to thank Drs. G. Carmody, H. Yamazaki, and L. Bonen for their advice. Special thanks are extended to the members of Vern's group for encouragement, useful criticism, and comradeship over the years, especially on weekends and late nights in the lab. Finally, thanks to all friends and relatives for putting up with me (or in most cases without me) during these demanding times.

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List of Abbreviations

BSA	bovine serum albumin
b.p.	base pairs
cDNA	complementary DNA; copy DNA
Ci	curie
cis-acting sites:	1. sites which control adjacent genes irrespective of the presence in the cell of other alleles; 2. any sequence of DNA that functions by being recognized rather than by being converted into a diffusible product. Also known as cis-dominant sites.
cpm	counts per minute
DEAE	diethylaminoethyl
Denhardt's	(1X) 0.02% Ficoll, 0.02% poly(vinylpyrrolidone), 10 mg/ml BSA
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
GDP	guanosine 5' diphosphate
h	hour(s)

induction: the effect whereby certain proteins are produced only when cells are grown on the appropriate substrates.

IPTG isopropylthiogalactoside

IVT in vitro translation

kbp kilobase pairs

min. minute(s)

mM millimolar

mRNA messenger RNA

Mr relative molecular weight

O.D. optical density

PAGE polyacrylamide gel electrophoresis

PEG polyethylene glycol

PIPES 1,4-piperazinediethanesulfonic acid

repression: the effect whereby the production of a protein is halted.

RF replicative form

RNA ribonucleic acid

RP reverse primer

r.p.m. revolutions per minute

SDS sodium dodecyl sulfate

SSC (1X) 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0

TCA trichloroacetic acid

TE 10 mM Tris-HCl pH 7.6, 1mM EDTA

trans-acting: specifying a product, usually protein, that can diffuse through the cell and act on all relevant sites in the cell, whether they are present on the same or different molecules of DNA.

Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UTR	untranslated region
U.V.	ultraviolet
X-gal	5-dibromo 4-chloro 3-indolylgalactoside

I. INTRODUCTION

A) Yeast and Fungi that Utilize Starch in Growth

Yeasts comprise some 39 genera with about 350 recognized species, and they are found in a wide range of terrestrial and aquatic habitats in which a suitable carbon source is available (Raven et al., 1976). From these numbers, it is known that approximately 150 species of yeast are capable of utilizing starch as a sole source of carbon for aerobic growth (McCann and Barnett, 1986). Addressing fungi in general, approximately 100 000 species have been described, and it is estimated that as many as 200 000 more may be awaiting discovery by mycologists (Raven et al., 1976). Among these species, starch is widely utilized as a carbon source.

Starch is a polymer of glucose which exists in two forms: amylose and amylopectin (Marshall, 1972). Amylose is comprised of long, unbranched chains of α -1,4-linked D-glucopyranose residues. These are poly-disperse chains which range in molecular weight from several thousand up to 500 000. In water, amylose forms hydrated micelles with individual molecules forming twisted, superhelical coils. Amylopectin is a highly branched polysaccharide, with branches averaging in length from 24-30 glucose residues. The backbone glycosidic linkage of amylopectin is made of α -1,4 bonds, while the branch points possess α -1,6 bonds. Amylopectin is also a micellular compound in the presence of

Water and its molecular weight may reach up to 100 million.

Next to cellulose, starch is probably the second-most abundant, renewable carbohydrate in nature. Starch is usually found as intricate, granules in plant seeds, roots, tubers, and stem piths; it can also be found in many plant-related lower eukaryotes (Swinkles, 1985). The ratios of amylose and amylopectin differ between starches from different plants and even from within the same plant (Fogarty and Kelly, 1979).

Organisms ranging from prokaryotes to humans produce enzymes capable of degrading starch (Mackay et al., 1985). As already mentioned, some (but not all) yeast and fungi possess various combinations of the amylolytic enzymes listed in Table 1. Amylose can undergo hydrolysis by α -amylase which randomly hydrolyzes α -1,4 glycosidic bonds to give a mixture of glucose multimers and free maltose. Maltose is not degraded readily by this enzyme. Amylose can also be hydrolytically digested by the action of β -amylase which cleaves successive maltose units beginning from the non-reducing end of the carbohydrate (Marshall, 1972). Maltose can in turn be digested to two glucose units by either the actions of α -glucosidase (maltase) or amyloglucosidase (glucoamylase).

The α - and β -amylases can also partially hydrolyze amylopectin. The polysaccharides of intermediate chain length remaining after the action of these enzymes on

Table I. Amylolytic enzymes found in some yeast and fungi.

<u>E.C.#</u>	<u>Name</u>	<u>Systematic Name</u>	<u>Reactions</u>
3.2.1.1	α -Amylase	α -1,4 Glucan 4 glucanohydrolase	Endohydrolysis of α -1,4-D-glucosidic linkages, liberating reducing groups with α -configuration.
3.2.1.2	β -Amylase	α -1,4 Glucan 4 maltohydrolase	Hydrolysis of alternate α -1,4-D-glucosidic linkages beginning at the non-reducing ends to form units of maltose.
3.2.1.3	Glucoamylase (Amylogluco- sidase)	α -1,4 Glucan glucohydrolase	Exohydrolysis of α -1,4-D-glucosidic linkages, successively from non-reducing ends of D-glucan, releasing β -D-glucose.
3.2.1.9	Pullulanase, (Isoamylase, Debranching enzyme)	α -1,6 Glucan 6 glucano- hydrolase	Hydrolysis of α -1,6-D-glucosidic branch linkages in isomaltose and dextrans.
3.2.1.20	α -Gluco- sidase, (Maltase)	α -1,4 Glucoside 4 glucohydrolase	Hydrolysis of terminal, non-reducing α -1,4-linked α -D-glucose residues of oligosaccharides with release of α -D-glucose.

amylopectin are classified as dextrans. α - and β -Amylases are both incapable of hydrolyzing the α -1,6 linkages found at the branch points of amylopectin. The end product of β -amylase hydrolysis on amylopectin is a large, highly branched core which is called the limit dextrin as it represents the limit of attack by β -amylase. The limit dextrans can be further digested by the action of debranching enzymes which are able to hydrolyze the α -1,6 glycosidic linkages found at the branch points (Sills *et al.*, 1982). Glucoamylase appears to be the most versatile of the amylolytic enzymes, not only cleaving terminal α -1,4 glycosidic bonds which result in the release of glucose from the non-reducing ends of starch chains, but (in most forms of the enzyme) cleaving α -1,6-glucosidic bonds when the adjacent bond is α -1,4 (Sills *et al.*, 1982; McCann and Barnett, 1986). Therefore, to completely degrade starch to mono and disaccharides such as glucose and maltose, a combination of hydrolytic enzymes must be present, with either a debranching enzyme or glucoamylase being a strict prerequisite.

B) Properties of Yeast and Fungal Glucoamylases

Glucoamylases have been identified in a variety of microorganisms, plants and animals (including humans) where it releases glucose from starch and glycogen (Manjunath *et al.*, 1983). However, at the molecular level little is known

about these enzymes in plants and animals.. Therefore the following discussion is limited to yeast and fungal glucoamylases.

Many of the general properties of yeast and fungal glucoamylases were reviewed by Manjunath et al., (1983). Glucoamylases tend to have a pH optima between 4 and 5, and a temperature optima between 40 and 60°C. It is not uncommon to find more than one form of the enzyme in an amylolytic microorganism. Glucoamylase needs no cofactors for activity or stability. On the average, the Mr of this enzyme class is between 48 kDa and 80 kDa and usually has no subunit structure. Table 2 lists the known Mr of yeast and fungal glucoamylases (see reviews by Manjunath et al., 1983; McCann and Barnett, 1986). While all known glucoamylases are glycoproteins, their amino acid composition varies considerably. The carbohydrate content of glucoamylases range from 3 to 30%. Most of the carbohydrate consists of mannose, however glucose, galactose, glucosamine and xylose have also been detected. The carbohydrate moieties are not believed to play a role in enzyme activity or antigenicity, but it is thought that they stabilize the enzyme by helping to maintain three dimensional structure in solution. At present the genes coding for the glucoamylases of Aspergillus niger/awamori (Nunberg et al., 1984; Svensson et al., 1983), Saccharomyces diastaticus (Yamashita and Fukui, 1983; Erratt and Nasim, 1986; Pretorius et al., 1986), S.

Table II. Molecular weights of some yeast and fungal glucoamylases.

Source (Fungal)	Glucoamylase I	Ref#	Glucoamylase II	Ref#
<u>Aspergillus awamori</u>	71 600	16	67 500	16
<u>A. candidus</u>	75 000	7	60 000	7
<u>A. foetidus</u>	75 000	6	54 000	6
	78 000	7	60 000	7
<u>A. niger-1</u>	79 000	7	72 000	7
<u>A. niger-2</u>	79 000	7	60 000	7
<u>A. niger-3</u>			60 000	7
<u>A. niger</u>	65 000	19	55 000	19
<u>A. oryzae</u>	69 000	10		
<u>A. phoenicus</u>	62 000	5		
<u>A. saitoi</u>	90 000	4	70 000	4
<u>Endomyces sp.</u>	55 000	3		
<u>Mucor rouxianus</u>	59 000	21	49 000	21
<u>Rhizopus delemar</u>	100 000	12		
<u>R. javanicus</u>	48 000	23		
<u>R. niveus</u>	74 000	20	58 600	20
<u>R. oryzae</u>	65 000	1		
Source (Yeast)	Glucoamylase I	Ref#	Glucoamylase II	Ref#
<u>Lipomyces kononenkoae</u>	81 500	17		
<u>Saccharomyces</u>	306 000	22	300 000	14
<u>diastaticus</u>	250 000	25	90 000	14
	150 000	8		
<u>Saccharomycopsis</u>	53 000	18		
<u>fibuligera</u>	55 000-58 000	18		
<u>Schwanniomyces</u>	155 000	9,24	117 000	15
<u>alluvius</u>			135 000	9
<u>Schwann. castelli</u>	146 000	2		
	99 000	11	45 000	11

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- 23) Watanabe and Fukimbara (1973)
- 24) Wilson and Ingledew (1982)
- 25) Yamashita et al. (1986)

Cerevisiae (Yamashita and Fukui, 1985), and Saccharomycopsis fibuligera (Yamashita et al., 1985) have been cloned. Similar work on genes from Schwanniomyces yeast amylolytic enzymes has not been done.

C) Expression and Regulation of Carbohydrate Metabolism in Yeast

Studies on the biochemical pathways involving specific carbohydrate metabolism such as the breakdown of galactose, maltose and sucrose have been extensively studied in S. cerevisiae, and have provided valuable information for the understanding of differential gene expression in yeast.

The model of galactose regulation in S. cerevisiae originally proposed (Douglas and Hawthorne, 1966) has been revised (Oshima, 1982). Expression of the structural genes of the Leloir pathway, GAL 7 (galactotransferase), GAL 10 (galactoeptimerase), and GAL 1 (galactokinase) as well as the galactopermease gene (GAL 2) are all positively regulated by the GAL 4 gene product and negatively regulated by the GAL 80 gene product (Douglas and Hawthorne, 1966). Both the GAL 4 and GAL 80 proteins are constitutively synthesized. In the absence of inducer (galactose), the GAL 80 protein interacts with the GAL 4 protein at a particular site (i.e. region(s) along the GAL 4 protein having affinity for GAL 80 protein interaction), preventing the latter, "positive" factor from activating expression of the Leloir pathway

genes (Oshima, 1982). Presence of inducer inhibits the function of the GAL 80 "negative factor" (or dissociates it from the positive GAL 4 factor), allowing the GAL 4 product to activate expression of the structural genes at the level of transcription. Glucose represses this system indirectly by either altering or destroying galactopermease, in turn preventing uptake of galactose into the cell. The genes GAL 7, GAL 10, and GAL 1 have been mapped to Chromosome II, GAL 2 to Chromosome XII, GAL 4 (and GAL 81) to Chromosome XVI, and GAL 80 to Fragment 8. The three structural genes GAL 7, 10, and 1 are clustered in a 7 kbp region (St. John and Davis, 1981), but are not transcribed as a polycistronic mRNA as in the lactose operon of the prokaryote E. coli (Freifelder, 1983). The GAL 1 gene is transcribed in a divergent direction from that of GAL 7 and GAL 10. However, the genes are coordinately regulated through a "cis" acting, enhancer sequence located in a 600 bp segment between GAL 1 and GAL 10 (St. John and Davis, 1981). GAL 7 and GAL 10 each appear to have separate transcription initiation sites and therefore promoters.

Maltose regulation in S. cerevisiae appears to be similar to galactose regulation, however distinct differences do exist (Hong and Marmur, 1986). There are five unlinked loci: MAL 1, MAL 2, MAL 3, MAL 4, and MAL 6. Presence of any of these loci confers on the yeast cell an ability to utilize maltose as a carbon source. Each MAL locus encodes

three gene products: MAL S (intracellular α -glucosidase), MAL T (maltose permease), and MAL R (a regulatory protein). Both of the structural genes (MAL S, MAL T) have been shown to be maltose inducible and glucose repressible under the coordinate regulation of the "trans" acting MAL R gene product at the level of transcription (Needleman *et al.*, 1984). In the case of MAL 6, the intergenic region between MAL 6S and MAL 6T contains the "cis" acting sites for binding of the positively acting regulatory protein (i.e. the MAL 6R gene product). The presence of maltose or glucose activates or represses the transcription of MAL 6S or MAL 6T from opposite strands, controlling the expression of these two structural genes (Cohen *et al.*, 1985). Maltose permease levels were shown to be sensitive to carbon catabolite repression by glucose (Siro and Lovgren, 1979).

Whether glucose preferentially limits the accumulation of MAL 6T mRNA over MAL 6S mRNA by carbon catabolite repression is unknown (Cohen *et al.*, 1985). All three MAL genes are located within an approximately 9 kbp stretch of DNA. The MAL 6S and MAL 6T open reading frames are located 800 bp apart and are transcribed divergently following induction by maltose. The clustered arrangement of a regulatory gene adjacent to the sequence it controls has not been observed in any other eukaryote and is reminiscent of bacterial operons except that the RNA molecules are not polycistronic (Needleman *et al.*, 1984).

The SUC gene family differs from the MAL and GAL gene families in that any one of the SUC genes (SUC 1-SUC 7) codes for two structural forms of invertase (a 60 kDa intracellular, nonglycosylated enzyme and a 62 kDa nascent enzyme, which after glycosylation is secreted as an 85 kDa enzyme) which are transcribed respectively by initiation at different 5' sites of the same gene (Chu and Maley, 1980; Carlson and Botstein, 1982; Brown *et al.*, 1984). While the intracellular, non-glycosylated invertase is constitutively synthesized, the secreted, glycosylated form is induced by sucrose and regulated by glucose repression, and this repression has been found to occur at the level of transcription and is regulated at the linked SNF 1 locus (Carlson and Botstein, 1982).

D) Expression Of Amylolytic Enzymes of Schwanniomyces

The budding yeasts of the genus Schwanniomyces are generally haploids, although diploidy through successive spore generations can be obtained by cellular fusion (James and Zahab, 1984) which may form one, or more rarely, two ascospores following meiosis (Phaff and Miller, 1984). Information on the genetics and physiology of the budding yeast Schwannniomyces is limited (Kurtzman *et al.*, 1972; Price *et al.*, 1978; Johannsen and van der Walt 1980; Wilson *et al.*, 1982a,b; Dhawale and Ingledew 1983b; Dowhanick *et al.*, 1984; James and Zahab 1984; Phaff and Miller 1984;

Lusena et al., 1985; Ingledew, 1987; Seligy et al., 1987) as most of the emphasis has been on the production and the characterization of its amylolytic enzymes (Clementi et al., 1980; Oteng-Gyang et al., 1981; Calleja et al., 1982; Frelot et al., 1982; Sills and Stewart 1982; Wilson et al., 1982; Dhawale and Ingledew 1983a; Calleja et al., 1984; Sills et al., 1984a,b; Simoes-Mendes 1984; Rossi and Clementi 1985). Schwanniomyces spp. are capable of completely hydrolyzing starch to glucose by producing extracellular α -amylase and glucoamylase (Sills and Stewart, 1982). The glucoamylase from Schwann. castellii is reported to have both α -1,4 and α -1,6 (debranching) activity (Sills and Stewart 1982; Sills et al., 1983b). However, two distinct extracellular glucoamylases, with different levels of debranching activity, have been isolated from Schwann. alluvius cultures as shown in Table II (Moranelli et al., 1982). Since these enzymes are labile under conditions similar to those used in the tunnel pasteurization process (61°C, 10 minutes) of the brewing industry (Oteng-Gyang et al., 1981; Sills and Stewart 1982; Wilson and Ingledew 1982b), they have been employed in a novel process that has been proposed for the production of low carbohydrate beer (Sills et al., 1983a,b).

Differential levels of amylolytic activity mediated by glucose regulation have been observed in Schwanniomyces spp. either by assaying for breakdown of starch or liberation of glucose from starch. In Schwann. castellii, amylolytic

activity can be repressed at glucose concentrations greater than 3 mM (Sills and Stewart, 1982). In Schwanniomyces spp. amylolytic activity is repressed in the presence of 2-deoxyglucose, an analogue of glucose which the wild type strains recognize as glucose, but fail to metabolize. This analogue was used to obtain derepressed mutants of α -amylase (but not glucoamylase) synthesis in Schwann. alluvius (Wilson and Ingledew, 1982). Ultraviolet (U.V.) and nitrosoguanidine mutagenesis were used to obtain 2-deoxyglucose resistant mutants of Schwann. castellii showing a three to six fold range of either hyperproduction or derepression of both α -amylase and glucoamylase (Dhawale and Ingledew, 1983a). U.V. irradiation and ethylmethane sulphonate (EMS) mutagenesis of a wild type Schwann. castellii strain resulted in the isolation of a 2-deoxyglucose resistant mutant of Schwann. castellii exhibiting constitutive α -amylase activity in glucose containing media (Sills et al., 1984b). In both the wild type and derepressed mutant strain, maltose was found to be a stronger inducer of α -amylase activity than starch. However, no difference in glucoamylase activity was detected from either strain under inducing conditions. Since both strains produce about one-third less the glucoamylase activity when grown in 1% w/v glucose, this indicated that a basal level of constitutive glucoamylase synthesis occurred (Sills et al., 1984b).

Two mutants of Schwann, occidentalis resistant to glucose repression were isolated in a similar manner (McCann and Barnett, 1986). These mutants degraded starch rapidly, but grew slowly on starch or its hydrolysis products, so that glucose accumulated in the medium. Sucrose inducible production of invertase was not repressed by glucose in these two mutants, as well as α -glucosidase (assessed by maltose hydrolysis) and isomaltase. Hexokinase activity was severely reduced in these mutants (about 16-40 fold) and it was hypothesized that hexokinase might be associated with a general regulatory system involving overall repression similar to that observed in hex 1 mutants of S. cerevisiae (Entian et al., 1977).

Recent studies by Lusena et al., (1985) on amylolytic activity of Schwann, alluvius indicated that constitutive levels of α -amylase and glucoamylase are synthesized and exported into medium during exponential growth in starch, after which export of amylolytic enzymes increases until a tenfold increase in activity is reached by late stationary phase. The appearance of extracellular amylolytic activity in the medium was also found to be oxygen dependent and glucose repressible. Cultures grown under oxygen limitation or in glucose medium showed no amylolytic activity on SDS-polyacrylamide gels.

To summarize, by the use of both wild type and mutant strains of Schwanniomyces, it has been determined that

amylolytic activity is induced by the presence of substrates such as maltose and soluble starch, and repressed in the presence of their limit hydrolysis product, glucose.

E) Research Objectives

Differential gene expression involving regulation of galactose, maltose, and sucrose in S. cerevisiae has been studied at the molecular level. The mechanisms of regulation of enzymes involved in carbohydrate metabolism in S. cerevisiae may be similar to those used to regulate expression of amylolytic enzymes in Schwanniomyces. However, since very little or nothing is known about the molecular genetics of this yeast, the main objective of this thesis was to obtain molecular information on amylolytic expression in Schwann. castelii. Through use of enzymatic assays and a polyclonal antibody specific to the major, active, 146 kDa extracellular glucoamylase, I determined the Mr of its precursor and major post-translational modification products. In addition, I examined the relative intracellular and extracellular abundance of the precursor and major post-translated glucoamylase derivatives in relation to various parameters such as growth and substrate and demonstrated that glucoamylase expression is controlled mainly at the level of transcription. The most striking feature of this yeast system was the rapid, induction of glucoamylase expression which occurred when cells were

transferred (shifted) to maltose media and its equally rapid repression when the same cells were shifted back to glucose. These results, which have now been published in part (Dowhanick et al. 1988), indicated that the mature 146 kDa glucoamylase is induced to about 100 fold over background within the first 30 minutes of transfer of cells from glucose to maltose media. Using this information, RNA believed to include maltose-induced transcripts was isolated and a cDNA library was constructed. This library was selectively screened by various DNA-DNA hybridization techniques to isolate clones containing sequence coding for message induced by maltose, but not by glucose. In the remainder of this thesis I report on the cloning and characterization of a maltose-inducible, glucose repressible gene (Scas-1). These results clearly show that the physical size of the putative open-reading frame and the pattern of induction and repression of Scas-1 when compared to the 120 kDa precursor glucoamylase, measured by in vitro translation, are virtually the same.

II. MATERIALS AND METHODS

A) Growth of Bacteria and Yeasts

a) Bacteria (Escherichia coli K12)

Strain HB101 (F-, hcdS20, r-b,m-b, recA13, ara-14, proA2, lacY1, galK2, rpsL20(Sm^r), xyl-5, mtl-1, supE44) was maintained on LB (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2) plates at 4°C. Strain JM101 (supE, thi, (lac-proAB), (F, traD36, proAB, lac^a, ΔAM15) was maintained on M9 (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 2mM MgSO₄, pH 7.4) plates at 4°C. Strain JM103 (Δlac pro, supE, thi, strA, sbcB15, endA, hspR4, F', traD36, proAB, lacI^a, ΔAM15) was maintained on M9 plates at 4°C. Strain JM109 (recA1, endA1, gyrA96, thi, hcdR17, supE44, relA1, (lac-proAB), (F', traD36, proAB, lacI^a, ΔAM15) was maintained on M9 plates at 4°C. Strain K802 (hcdR+, hcdM+, gal-, met-, supE) was maintained on NZY (1.0% casamino acids, 0.2% MgCl₂, 0.5% NaCl, 0.5% yeast extract) plates at 4°C.

b) Yeasts

Schwanniomyces castellii strain 1402 (Labatt Culture Collection) and Schizosaccharomyces pombe strain 2448 (h-, leu 1-179 - N.R.C.C. Culture Collection) were maintained on 2% glucose, peptone yeast agar slopes at 4°C. Cells were grown in minimal media containing 0.67% (w/v) Bacto yeast nitrogen base without amino acids (Difco) supplemented with either 1% (w/v) glucose (Anachemia), 1% (w/v) maltose (Sigma) or 1% (w/v) soluble starch (Fisher).

c) Induction Experiments

Cells were inoculated into 250 ml Erlenmeyer flasks containing 100 ml minimal medium + 1% glucose to an initial concentration of 2×10^6 cells/ml and grown for 24 hours at 30°C in a New Brunswick gyratory shaker at 160 r.p.m. One ml of the 24 hour culture was reinoculated into 100 ml of minimal medium + 1% glucose and grown under similar conditions. This reinoculation was repeated in order for cells to become thoroughly adapted to the medium. To obtain exponentially growing cells, the culture was harvested by centrifugation at 8000 r.p.m. at room temperature and the cell pellet resuspended in 10 ml minimal medium + 1% glucose. Five ml were then inoculated into 100 ml minimal medium + 1% glucose and grown under conditions already discussed. The culture was monitored by O.D._{600nm} readings (using a Coleman 295 spectrophotometer) until exponential growth had been established (approximately 2 hours). The cells were then pelleted (8 000 r.p.m., 5 minutes, room temperature) and washed twice at room temperature with a minimal salts medium consisting of (per litre): 0.1 g l-aspartic acid, 1.0 g NH_4Cl , 2.0 g KH_2PO_4 , 0.3 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 12 mg ZnCl_2 , 5 mg FeCl_2 , 1.5 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.1 mg thiamin in order to remove any remaining extracellular glucose. Cells were pelleted and resuspended in an equal volume of minimal salts medium. This slurry was then added to 15 ml of minimal medium in 125 ml side arm Erlenmeyer

shake flasks until the desired cell concentration, as indicated by O.D._{600nm} readings zeroed against medium without cells, was obtained. The shake flasks were then transferred to a New Brunswick shaker and incubated at 30°C, 160 r.p.m. At specific times, samples were removed in order to analyse cell-associated (cell lysate) and extracellular (culture supernatant) proteins (see B)d)) as well as extracellular glucoamylase activity (see B)b)). In the analysis of the effects of different substrates on glucoamylase activity, cellular growth was monitored by O.D._{600nm} at the respective sampling times.

d) In Vivo Labelling of Proteins with ³⁵S-Methionine and Cell Fractionation

Strain 1402 cells were prepared and shifted from non-inducing to inducing medium as described in A)c) except that the final volumes of the cultures were 10ml and 100 µCi of ³⁵S-methionine (Amersham) were added to the minimal supplemented media. One ml samples were removed at 15, 30, 45, and 60 minutes, and 2, 3, 4, 5, and 24 hours after reinoculation. Total cell associated and extracellular proteins were separated from each other and prepared (see below). Samples were electrophoresed in 10% separating, 4% stacking polyacrylamide gels for 16 hours at 45 volts in Laemmli buffer (see B)d)). The gels were then fixed in 30% methanol, 10% acetic acid for 1 hour, Enhanced (Dupont) for

1 hour, and washed in deionized distilled water for 1 hour. The gels were dried on a BioRad 224 gel slab dryer at 60°C for 2 hours and exposed to Kodak X-Omat AR film at -80°C. Culture samples of 0.5 ml were transferred at various times into 1.5 ml microfuge tubes and centrifuged at 13 000 r.p.m. for 2 minutes. The supernatants were transferred separately to other tubes where 25 µl was removed and immediately used for the glucoamylase assay (see B)b)). The remainder of the supernatant was made 2 mM with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and stored at 4°C until all samples had been taken. The pellets were immediately flash frozen in a dry ice/methanol bath until all samples had been collected. Fifty microliter aliquots of the extracellular protein-containing supernatants were boiled for 2 minutes with an equal volume of 2X Laemmli buffer, before submitting to SDS polyacrylamide gel electrophoresis (see B)d)).

Cell-associated proteins were extracted from the frozen pellets by adding an equal volume of sterile glass beads (0.2 mm diameter) along with 2 volumes of Buffer B (50 mM Tris, pH 7.5, 5 mM EDTA, pH 8.0, 150 mM NaCl, 0.05% Nonidet P-40, Willick *et al.* 1984). The mixture was vortexed at high speed for 2 minutes and then centrifuged at 13 000 r.p.m. for 2 minutes. The supernatant (now containing cell-associated proteins) was made 1X in Laemmli buffer, placed in a boiling water bath for 5 minutes and electrophoresed in

polyacrylamide gels (see B)d)).

B) Enzyme Characterization

a) Purification and Antibody Production

Anti-glucoamylase antisera was obtained from New Zealand white rabbits and partially-purified as described by Willick et al. (1984). The active glucoamylase used as antigen was purified as a single 146 KDa polypeptide from clarified supernatants of starch grown cells using a Mono Q (Pharmacia) column followed by TSK chromatography (G. Willick, J.R. Barbier and P. Zygora, unpublished).

To test specificity of the anti-glucoamylase antisera against in situ glucoamylase activity, supernatant from 4 hour maltose-induced cells (containing active extracellular glucoamylase) was competed with increasing amounts of antisera as follows: 30 μ l of supernatant was combined with 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , 1, 2, 5, and 10 μ l of antisera in 3 ml citrate-phosphate buffer pH 5.5 for 30 minutes at room temperature. The liquid assay for glucoamylase activity was then performed on each sample as described in B)b).

b) Liquid Assays for Activity

The liquid assay used for the determination of glucoamylase activity is based on the determination of liberated glucose, which is estimated enzymatically using glucose oxidase-peroxidase. The hydrogen peroxide formed during the dehydrogenation of glucose by glucose oxidase is

used by peroxidase to oxidize dimethoxybenzidine to a brown colour, which is measured at O.D.436nm.

Although many substrates such as amylose, amylopectin, amyloextrins, maltose, p-nitrophenyl- α -D-glucoside, and glycogen have been for this assay, the most commonly used substrate (and the one used in these experiments) is starch (Wilson and Ingledew 1982 and Sills *et al.* 1984a). Supernatants (30 μ l) were combined with 150 μ l of 0.5% w/v starch solublized by boiling in citrate-phosphate buffer pH 5.5 for 1 minute. Three ml of citrate-phosphate buffer pH 5.5 were added and the mixture was incubated for 30 minutes at 40°C. Three ml of glucose oxidase reaction buffer (per liter: 14.72 g NaH_2PO_4 , 6.47 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.04 g peroxidase, 0.07 g dimethoxybenzidine, and 4.00 ml glucose oxidase (Sigma-added just before use) were then added and allowed to react for 4 minutes at room temperature before O.D. readings were taken at 436 nm. These reactions were

zeroed against a blank using 30 μ l of sterile water in place of supernatant to compensate for any free glucose liberated during initial boiling of the starch. Also, the amount of free glucose present in the extracellular supernatant prior to addition of soluble starch was measured by adding 150 μ l of water to the 30 μ l samples, instead of soluble starch. The difference between the free glucose present before and after addition of soluble starch represented the amount of glucose liberated due to the activity of glucoamylase on the substrate during the 30 minute incubation at 40°C. All experiments were performed a minimum of three times with results not varying by greater than 10 percent.

c) In Situ Assays for Activity

Starch is composed of amylose and amylopectin, neither of which are truly soluble in water. In the presence of water, these components tend to coil and form micelles. The core of the coils can accommodate iodine molecules. The formation of an iodine-amylose complex gives a dark blue colour while an amylopectin-iodine complex gives a red-violet colour (Baum and Scaife, 1975; Lehninger, 1975). Thus, hydrolysis of starch due to the secretion of an amylolytic enzyme can be visually monitored by the presence of a "clearing" on a starch plate exposed to iodine. Amylolytic activity could be detected by growing cells on solid media containing soluble starch and testing for starch

hydrolysis by exposing the plates to iodine vapours. Cells were plated and grown for 2 to 4 days at 30°C on minimal + 1 to 2% soluble starch (Fisher). The plates were then either stored at 4°C or exposed to iodine vapour immediately after incubation. In the case of yeast, minimal medium + 1-2% soluble starch was generally used for detection of extracellular activity. In the case of E. coli, LA plates (see F)e) supplemented with 1% soluble starch + 1 mM IPTG was used.

d) Immunological Detection Studies

Prior to immunoanalysis, the cell-associated and extracellular samples were electrophoretically separated (as described in this section) and resolved using a modified silver stain technique of Goldman et al., (1981). The gel was soaked for 30 minutes in i) 50% methanol/10% acetic acid, ii) 5% methanol/7% acetic acid, iii) 5% glutaraldehyde. The gel was rinsed in large volumes of H₂O for a minimum of 2 hours, and then immersed in 5 µg/ml DTT in H₂O for 30 minutes. The DTT solution was decanted and replaced with 0.1% w/v silver nitrate for 30 minutes. The gel was rinsed briefly in H₂O and then floated in developer (50 µl of 100% formaldehyde/100 ml of 3% Na₂CO₃). Developing was stopped (usually after 5-15 minutes) by addition of 1/10th volume of 2.3 M citric acid. After rinsing briefly in H₂O, the gel could be stored moist or

photographed using fluorescent transillumination.

Immunoprecipitation of cell-associated and extracellular glucoamylase (including the in vivo labelled total proteins described in A)d)) was carried out using 60 μ l (approximately 25% of the respective cell-associated and extracellular protein) combined with 330 μ l REPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 0.1% SDS, 1% Na deoxycholate, 1% Triton X-100, 1 mM PMSF) and 10 μ l glucoamylase antisera. The mixtures were slowly rotated overnight at 4°C. Forty microliters of a slurry of Protein A-Sepharose (Pharmacia), was added to each sample and rotated for 2 hours at 4°C. The mixture was centrifuged (3 min./13 000 r.p.m.) and the pellet was washed 5 times with 1 ml REPA buffer. The pellet was resuspended in 40 μ l REPA buffer along with 40 μ l 2X Laemmli buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue), boiled for 5 minutes and repelleted. The supernatant was loaded onto a 10% separating/4% stacking polyacrylamide gel according to the method of Laemmli (1970). The separating and stacking gels were prepared from a stock solution of 30% w/v acrylamide (BDH) and 0.8% N,N'-bis-methylene acrylamide (BDH). The final separating gels contained 375 mM Tris-HCl pH 8.8, and 0.1% SDS, while the final stacking gels contained 125 mM Tris-HCl pH 6.8 and 0.1% SDS. The gels were polymerized chemically by the addition of 0.025% v/v tetramethylethylenediamine (TEMED)

and 0.025% v/v ammonium persulphate. The electrode buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS, adjusted to pH 8.3. The samples were loaded after boiling and electrophoresed for 16 hours at 45 volts. The gel was then fixed in 30% methanol/10% acetate for 30 minutes at room temperature, treated with either En³Hance (DuPont) or Amplify (Amersham) for 30 minutes (room temperature). En³Hance-treated gels were washed in water for 30 minutes at room temperature. The gels were dried on a BioRad 224. gel slab dryer at 60°C for 2 hours and exposed to Kodak X-Omat AR film at -80°C.

Cell-associated and extracellular proteins were also analysed by the Western blotting method of Towbin et al. (1979). Proteins subjected to SDS PAGE were electrophoretically transferred to nitrocellulose (Schleicher and Schuell) for 2 hours at 40 mA in Western transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% v/v methanol, pH 8.3. Upon completion of transfer, the filter was suspended in fresh Western transfer buffer until immunodetection was performed (usually the same day).

Immunodetection of the filters was performed using either ¹²⁵I-protein A or anti-rabbit IgG alkaline phosphatase conjugate. The ¹²⁵I-protein A immunodetection assay was a modified procedure of Towbin et al., (1979) which is described as follows: each filter was washed for 10 minutes at 23°C in TBS buffer (500 mM NaCl, 0.05% v/v Tween 20, 20

1 mM Tris HCl pH 7.5, 20% v/v fetal calf serum (FCS) (Flow Laboratories). Filters were washed with 20 ml TBS for 2 minutes at 23°C before addition of antiglucoamylase antisera (50 μ l/20 ml TBS) and gentle rocking for 2 hours. The filters were then washed once in 20 ml TBS/FCS for 5 minutes followed by 3 washes in 20 ml TBS for 5 minutes. The filters were washed with 20 ml TBS/FCS for 5 minutes followed by 3 washes in 20 ml TBS for 5 minutes. The filters were immersed in 20 ml TBS/FCS containing 125 I (ICN) protein A (McConahey and Dixon 1980) at 1×10^6 c.p.m./filter for 20-30 minutes with gentle rocking, followed by successive washes, once in TBS/FCS for 5 minutes and thrice in TBS. Air dried filters were then exposed to Kodak X-Omat AR film for 16 hours at -80°C. Binding of glucoamylase antisera (50 μ l) with non-induced total cell-associated protein (5 μ l from 10 time points in Buffer B) was carried out by mixing with 100 μ l of Buffer B for 2 hours at 4°C before use.

The alkaline phosphatase immunodetection assay followed the ProtoBlot Immunoscreening System (Promega Biotech) as suggested by the manufacturer. All reactions were performed at room temperature. Non-specific protein binding sites were saturated by floating the Western blot in TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) + 1% BSA for 30 minutes. The blocking solution was replaced with primary (anti-glucoamylase) antibody in TBST (1:40 000 dilution) and the filter was incubated for 30 minutes. The

membrane was washed 3 times (5-10 minutes /wash) in TBST buffer to remove unbound antibody and then transferred to TBST containing a 1:7 500 dilution of anti-IgG alkaline phosphatase conjugate for 30 minutes. The filter was washed 3 times in TBST to remove unbound secondary antibody, and then transferred to the colour development solution consisting of 66 μ l NTB substrate (nitro blue tetrazolium) and 33 μ l BCIP substrate (5-bromo-4-chloro-3-indolyl phosphate) per 10 ml AP buffer (400 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The colour reaction was stopped by rinsing the membrane in distilled, deionized water for several minutes and then photographed while still moist.

C) Preparation and Characterization of RNA

a) RNA Extraction and Purification

Total RNA was isolated by modification of the procedures of Glisin et al. (1974) and Chirgwin et al. (1979). Cells were harvested by centrifugation for 2 minutes at 8 000 r.p.m. at room temperature. The supernatant was decanted and the cell pellet was immediately frozen in a dry ice-methanol bath. To a 0.5-1.0 g pellet (wet weight) of frozen cells, the following was added: an approximately equal volume of glass beads (0.5 mm diameter), 20 μ l 100% Sarkosyl, 20 μ l antifoam A (Sigma), 4.0 ml Guanidinium thiocyanate (GuCNS) solution (4.0 M GuCNS, 25 mM citrate, 1 mM EDTA, pH 7.0), and 40 μ l 14.1 M mercaptoethanol. The

mixture was then vortexed at high speed for 2 minutes and immediately placed on ice until all samples had been similarly treated. The samples were centrifuged at 8 000 r.p.m. and 4°C for 10 minutes (or until all debris had pelleted) and the supernatant was layered onto a 1.0 ml bed of 5.7 M cesium chloride (CsCl), 0.1 M EDTA, pH 7.0 in a Beckman ultracentrifuge tube. The RNA was pelleted for 16 hours at 30 000 r.p.m. and 18°C in a SW 50.1 rotor.

The supernatant and remaining debris were removed by aspiration and the centrifuge tube quickly inverted to allow supernatant to drain away from the clear RNA pellet at the bottom of the tube. Ethanol was added to the pellet before it was transferred into a 1.5 ml Eppendorf microfuge tube. The RNA precipitate was pelleted and the ethanol was removed. The pellet was resuspended in diethylpyrocarbonate (DEP-0.01%)-treated water, made 3 M with NH₄Acetate, and reprecipitated with ethanol. The dried RNA pellet was resuspended in DEP-treated water and optical density readings were taken at 260 nm. - Intact RNA was quickly confirmed by horizontal gel electrophoresis of samples in a 2.0% agarose, 0.025 M citrate gel (pH 3.5) stained (after electrophoresis) with 0.5 µg ethidium bromide (EtBr) and photographed using short wave ultraviolet light.

b) RNA Fractionation by Oligo dT Chromatography

Poly A⁺ RNA was separated from total RNA for cDNA

synthesis by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972) as described in Maniatis et al., 1982). Oligo(dT)-cellulose was equilibrated in loading buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1mM EDTA, and 0.1% SDS) and a 1.0 ml column was poured into a Pasteur pipette with siliconized glass wool at the base. The column was washed with 3 column-volumes of: a) sterile H₂O, b) 0.1 M NaOH + 5 mM EDTA, c) sterile H₂O. With the pH of the column less than 8, it was washed with 5 volumes of sterile loading buffer. Total RNA (1-2 mg) dissolved in sterile H₂O was heated to 65°C for 5 minutes. An equal volume of 2X loading buffer was added to the RNA and the sample was cooled to room temperature. The sample was then applied to the column. The flow-through was reheated to 65°C, cooled and reapplied to the column. The column was washed with 10 column-volumes of loading buffer. Poly A+ RNA was then eluted with 5 column volumes of elution buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.05% SDS). Three hundred μ l fractions were collected and OD_{280nm} readings were taken. Samples containing RNA received ammonium acetate (10 M stock) to a final concentration of 0.3 M. These samples were precipitated with 2.5 volumes EtOH, and the pellets were washed in 70% EtOH. The pellets were dissolved in sterile water. Approximately 25 μ g of poly A+ RNA was collected/mg total RNA.

c) In vitro Translation of RNA

Twenty μg aliquots of total RNA were translated in vitro using a rabbit reticulocyte lysate (Amersham). The reactions were carried out in a mixture containing: 80% v/v lysate, 0.75 $\mu\text{Ci}/\mu\text{l}$ ^{35}S -methionine (Amersham), 1 $\mu\text{g}/\mu\text{l}$ RNA, 94 mM KCl, and 1.4 mM MgCl_2 . The reactions were incubated at 30°C for 1 hour and then either stored at -70°C or immediately used.

Immunoprecipitation of in vitro translation products was carried out as described in B)d) using 50% (10 μl) of the IVT reaction in 330 μl REPA buffer. To the remaining sample, an equal volume of 2X Laemmli buffer was added, and the mixture was boiled for 5 minutes. The sample was pelleted and the supernatant was electrophoresed in 4% stacking, 10% separating SDS polyacrylamide gels for 16 hours at 45 volts in Laemmli buffer as described in B)d).

d) Quantitation of Specific RNA by DNA Hybridization

For Northern analyses, total RNA samples were loaded (20 $\mu\text{g}/\text{lane}$) onto 1% agarose, 2.2 M formaldehyde gels (Lehrach et al. 1977; Maniatis et al. 1982) and electrophoresed for 16 hours at 50 volts. The gel was washed in large volumes of water for 2 hours to remove formaldehyde. The RNA was transferred to nitrocellulose membrane using 3 M NaCl-0.3 M trisodium citrate (20X SSC) essentially as described for transfer of DNA by Southern (1975) with modifications

according to Thomas (1980). The gel was placed over two sheets of Whatmann 3 MM saturated with 20X SSC. The nitrocellulose membrane was wetted in 65°C H₂O, laid over the gel, and covered with 3 sheets of Whatman 3 MM, a 5-7 cm layer of paper towels (Swan), a glass plate, and a weight. Transfer of RNA was allowed to proceed overnight. The blot was air dried at room temperature and then baked in a vacuum oven for 2 hours at 80°C. The blot was then either stored indefinitely or prehybridized for 6-20 hours at 42°C in prehybridization/hybridization buffer consisting of: 50% v/v deionized formamide, 5X SSC, 50 mM sodium phosphate pH 6.5, 1X Denhardt's, and 250 µg/ml denatured salmon sperm DNA. Denatured, radiolabelled probe was added to fresh buffer, and the blot was hybridized for 20-24 hours at 42°C in a sealed plastic bag. The blot was washed with 2 changes of 2X SSC + 0.1% SDS for 10 minutes each at room temperature, and 2 changes of 0.1X SSC + 0.1% SDS for 15 minutes each at 50°C. The membrane was sealed moist in a plastic bag and exposed to X-ray film at -80°C.

D) Preparation and Characterization of DNA from Bacteria and Yeasts

a) Preparation of Plasmid and M13(RF) DNA

Plasmid and M13(RF) phage DNA were isolated by the rapid alkaline mini-plasmid procedure of Birnboim and Doly (1979). In this procedure, the bacterial are lysed by treatment with

lysozyme (2 mg/ml), 1% SDS, and 0.2 M NaOH. At high alkaline pH, the double strands of the bacterial chromosomal DNA are denatured and separated while the supercoiled plasmid or phage DNA remains linked. Neutralization with 3 M sodium acetate restores the double stranded nature of the supercoil DNA, but not the linear DNA. M13 RF-DNA was extracted from pellets of either JM103 or JM109 cells grown for 16 hours from an initial infection of 100 μ l exponentially growing cells with 50 μ l of plaque pure M13 stock (at 1×10^8 pfu/ml). Plasmid DNA was extracted from 10 ml to 100 ml cultures of either HB101, or JM103 transformants grown in the presence of 30 μ g/ml ampicillin. When required, ultra-pure plasmid or RF-DNA was obtained by subjecting the DNA preparations to cesium chloride ultra-centrifugation with ethidium bromide (Maniatis et al., 1982).

b) Preparation of M13 SS-DNA

Single stranded ("+" DNA) M13 DNA (also known as template) was isolated using the procedure of Messing (1983). Single plaques of M13 phage were incubated for 16 hours in a 50 ml Erlenmeyer containing 10 ml of 2YT medium inoculated with exponentially growing JM103 or JM109 cells. The cells were pelleted (8 000 r.p.m., 15 minutes, 4°C) and the phage remaining in the supernatant were precipitated with addition of 1/10th volume of 20% PEG, 2.5 M NaCl mixed at 4°C for 15 minutes. The precipitate was pelleted (8 000

r.p.m., 15 minutes, 4°C) and the supernatant was removed. The pellet was then dissolved in 500 μ l STE buffer (100 mM NaCl in TE) and phage DNA was extracted once with phenol (v/v), and twice with phenol/chloroform/isoamyl alcohol (50:48:2, v/v). An equal volume of 5 M NH_4 Acetate was added and the sample was EtOH precipitated twice. The samples were washed in 70% EtOH, dried and redissolved in sterile water at 200 ng/ μ l.

c) Preparation of λ Charon 4A Phage DNA

λ Charon 4A phage DNA (with and without Schwanniomyces inserts) were propagated by the confluent plate method of Slightom et al., (1980). DNA was extracted directly from PEG precipitated phage or from phage purified through a cesium chloride step gradient (Davis et al., 1979).

d) Preparation of Yeast DNA

Genomic DNA was isolated using a modification of the procedure of Cryer et al. (1975). Cells were inoculated into 2L Erlenmeyer flasks containing 1L minimal medium + 2% glucose to an initial concentration of 2×10^6 cells/ml and grown for 24 hours at 30°C in a New Brunswick gyratory shaker at 160 r.p.m. The culture was harvested by centrifugation at 8 000 r.p.m. at room temperature and cells were added to 1L minimal + 2% glucose medium. The culture was monitored by O.D._{600nm} readings until exponential growth had been established (approximately 2 hours). The cells

were then pelleted (8 000 r.p.m., 5 minutes, room temperature) and washed once in minimal salts medium (as described earlier) to remove the growth medium. The cells were resuspended in 20 ml of 1 M sorbitol, 20 mM DTT, 50 mM EDTA pH 7.5, and incubated for 15 minutes at room temperature. The cells were again pelleted and resuspended in 20 ml of 1 M sorbitol, 50 mM EDTA pH 7.5. Zymolyase 60 000 (Kirin) was added to a final concentration of 50 μ g/ml and the mixture was incubated at 37°C. Spheroplasting was monitored every 10 minutes by the clearing of 100 μ l of the mixture in 1 ml of a 5% SDS solution. Spheroplasts were pelleted (3 000 r.p.m., 10 minutes, 4°C) and resuspended in 10 ml TE buffer supplemented with 50 μ g/ml proteinase K and 1% SDS. The lysate was incubated for 1 hour at 37°C, 1 hour at 55°C and for 16-20 hours at room temperature. The lysate was phenol extracted twice, and phenol/chloroform/isoamyl alcohol (50:48:2) extracted once (8 000 r.p.m., 20 minutes, 4°C). The aqueous phase was adjusted to 2.5 M NH_4Ac final and EtOH precipitated. The precipitate was spooled onto a Pasteur pipette and then washed in 70 % EtOH before being dried and resuspended in TE buffer. An O.D._{260nm} reading was taken to determine the DNA concentration and the sample was tested for size by gel electrophoresis.

e) Characterization of DNA by Restriction Endonuclease Analysis

DNA from various sources were limit digested with a variety of restriction endonucleases using the high, medium or low salt buffers as described by Maniatis et al (1982). The samples were digested for 1-2 hours at 37°C with enzyme units per DNA (μ g) varying from 0.5-10 depending on whether the DNA was cloned or total genomic. The digests were terminated by the addition of 1/10th volume of stop buffer (100 mM EDTA, 0.5% SDS, 25% glycerol, 0.1% BPB) followed by heating at 55°C for 3-5 minutes. The samples were then either electrophoresed or stored at 4°C.

Horizontal gel electrophoresis of DNA was carried out using slabs of variable sizes of agarose (Seakem) as the supporting matrix (0.7-1.4% w/v) in either TAE (25 mM Tris, 25 mM acetic acid, 10 mM EDTA, pH 7.5) or TBE (10 mM Tris, 10 mM boric acid, 10 mM EDTA, pH 8.0) with or without the dye ethidium bromide (Sigma) at a final concentration of 0.5 μ g/ml (Maniatis et al., 1982). Samples were electrophoresed at approximately 5 volts/cm for various times. The migratory patterns of the DNA fragments within the gels were recorded by photography using a red filter and clear Polaroid screen to detect the ethidium bromide fluorescence by ultraviolet light at 310 nm. DNA molecular weight markers included Lambda wild type digested with EcoRI, HindIII separately or together, and pBR322 digested with

HinfI. In some cases the DNA markers were radiolabelled by a 3' fill in reaction using PolI Klenow and α - ^{32}P -dATP (Maniatis et al., 1982). Labelled DNA was detected by autoradiography after fixing the agarose gels in 7% TCA, drying on Whatman 3MM paper (as a support) with a slab gel dryer at 70°C, and exposing to X-Ray film at -70°C as required.

f) Characterization by DNA-DNA Hybridization

For Southern analyses, limit digested genomic DNA was transferred to Biodyne (Pall) nylon membrane using the alkaline procedure of Chomczynski and Qasba, (1984) with modifications. Upon completion of agarose gel electrophoresis of DNA (see D)c)), the ethidium bromide-stained gel was photographed using incident short-wave ultraviolet illumination (see D)c)). The DNA was partially hydrolyzed through acid depurination by soaking the gel for 15 minutes in 0.25 M HCl (Wahl et al., 1979), followed by denaturation for 30 minutes in 0.4 M NaOH, 0.6 M NaCl. The DNA was transferred (by passive diffusion) onto the Biodyne membrane for 4 hours using 0.4 M NaOH, 0.6 M NaCl as the transfer buffer. The filter was neutralized by placing (DNA side facing up) on a sheet of Whatman 3MM that had been wetted with 1 M Tris pH 7.0, 1.5 M NaCl for 2-3 minutes. The filter was air dried for 30 minutes and then baked for 1 hour at 80°C. The membrane was irradiated (DNA side up) for

5 minutes with short wave U.V. in order to crosslink the DNA to the solid support, and then either stored at room temperature or used immediately.

Filters were prehybridized at the appropriate temperature (depending on the stringency of the hybridization - see below) for approximately 2 hours by sealing in a plastic bag (Seal a Meal) containing prehybridization buffer consisting of: 6X SSC, 1X Denhardt's, 0.5% SDS, 100 μ g/ml denatured salmon sperm DNA and 0.05% sodium pyrophosphate. The DNA blot was then transferred to hybridization buffer (6X SSC, 1X Denhardt's, 20 μ g/ml tRNA and 0.05% sodium pyrophosphate) containing probe and incubated for 16-20 hours at the appropriate temperature. Depending on the stringency of the hybridization (see below), the filters were washed, sealed (moist) in a plastic bag, and exposed with an intensifying screen at -80°C as required.

<u>Stringency</u>	<u>Prehybridization</u>	<u>Hybridization</u>	<u>Washing Conditions*</u>
low	42°C	42°C	6X SSC/0.1% SDS
medium	50°C	50°C	2X SSC/0.1%SDS
high	65°C	65°C	0.1X SSC/0.1%SDS

* Filters were immersed for 1 minute in hybridization buffer at the respective hybridization temperature prior to washes, and in 6X SSC/0.05% sodium pyrophosphate for 1 minute after washes to remove excess SDS. All washes were repeated 3X for 10 minutes.

E) Synthesis of Complementary DNA (cDNA)

a) Single Strand cDNA for Competitive Hybridization Experiments

Upon completion of first strand cDNA synthesis (see E)b)), unincorporated deoxyribonucleotides were removed by passing the sample through two Sephadex G-50 spin columns (Maniatis et al., 1982). RNA was removed by addition of NaOH to 50 mM and incubation at 65°C for 60 min. The sample was then neutralized with HCl and buffered by addition of Tris pH 7.0 to 0.3 M (final).

b) Double Strand cDNA for Clone Construction

Double stranded cDNA was synthesized using the procedure of Gubler and Hoffman (1983) with modifications. For first strand synthesis, poly A⁺ RNA from 30, 60, and 90 minute maltose induced strain 1402 cells was denatured in 10 mM methylmercury hydroxide for 10 minutes at 23°C. The mercury was inactivated with 2 μ l of 700 mM 2-mercaptoethanol (Maniatis et al., 1982) and the following was added: 10 μ g oligo-dT, cDNA buffer (100 mM Tris-HCl pH 8.3, 10 mM MgCl₂ and 250 mM KCl, final) 1 mM (final) of each of dATP, dCTP, dGTP, and dTTP, 10 μ Ci α^{32} P-dATP (3 000 Ci/mMol) and 50 units of AMV reverse transcriptase (Life Sciences). First strand synthesis reaction proceeded for 30 minutes at 37°C and was monitored by Cerenkov counting of TCA-precipitable radioactivity (Berger, 1984).

The second strand was synthesized in a final volume of 100 μ l by adding one volume of nick translation buffer (50 mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 20mM KCl, and 5mM DTT), 1 unit RNase H (BRL), and 25 units of E. coli DNA polymerase I (BRL) as described by Rutledge (1987). After 2 hours incubation at 15°C, the double stranded cDNA was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (50:48:2), passed through a Sephadex G-50 spin column (Maniatis et al., 1982) to separate unincorporated and small incomplete reaction products, adjusted to 2.5 M NH_4Ac , and EtOH precipitated. The precipitate was pelleted, washed in 70 % EtOH, repelleted, dried, and resuspended in water to a final concentration of 10 ng/ μ l. A fraction of the sample was monitored by Cerenkov counting and by autoradiography after electrophoresis in a 1.4% agarose TAE gel.

F) Cloning of cDNA and Genomic DNA a) cDNA Cloning using the M13 Vector

The cloning of blunt-ended double stranded cDNA synthesized from maltose-induced message involved the use of M13 .mp19 RF vector limit digested at the single SmaI site (Yanisch-Perron et al., 1985). Ten μ g of vector was digested with 50 units of SmaI (BRL) in 50 μ l of 10 mM Tris-HCl pH 8.0, 20 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT for 2 hours at 37°C. The flush ends were dephosphorylated using 1 unit

of bacterial alkaline phosphatase (BAP) for 1 hour at 65°C. The vector was purified by incubation with proteinase K (1 µg/µl) for 30 minutes at 37°C followed by three extractions with phenol/chloroform/isoamyl alcohol (50:48:2) and precipitation with 2.5 volumes of EtOH in 2.5 M NH₄Ac. The pelleted vector was dried and resuspended in TE buffer at a final concentration of 10 ng/µl. The final ligation mixture included 100 ng vector and 10 ng cDNA in a volume of 20 µl containing 66 mM Tris-HCl pH 7.5, 5 mM DTT, 6.6 mM MgCl₂, 0.1 µg/µl BSA, and 1 mM ATP. Three units of T4 ligase (BRL) were added and the ligation mixture was incubated for 16 hours at 4°C.

E. coli strain JM109 (Yanisch-Perron et al., 1985) was transfected with the ligation mixture. Cells were made competent by modification of the method of Hanahan (1983). Approximately 3 µl of cells were removed from an M9 glucose minimal plate (Miller, 1972), and grown (16 hours, 37°C, 300 r.p.m.) in a 50 ml Erlenmeyer flask containing 10 ml of 2YT medium (1.6% Bacto tryptone, 1.0% Bacto yeast extract, and 0.5% NaCl). One hundred µl of cells were transferred to 25 ml 2YT in a 125 ml Erlenmeyer flask and further grown to a density of 0.6 to 0.7 O.D._{500nm}. The culture was harvested (5 000 r.p.m., 5 minutes, 4°C) and the pellet was suspended in 8 ml TFB (10 mM 2-N-morpholinoethane sulphonic acid pH 6.2, 100 mM RbCl, 45 mM MnCl₂.4H₂O, 10 mM CaCl₂.H₂O, 3 mM HA CoCl₂) and placed on ice for 15 minutes. The cells were

similarly pelleted and resuspended in 2 ml TFB before 70 μ l of fresh DMSO was added and left on ice for 10 minutes. Seventy μ l of 2.25 M DTT were gently swirled into the mixture and incubated for 10 minutes on ice before an equal volume of DMSO was added and left on ice for 5 minutes. Two hundred μ l of cells were gently swirled with 10 μ l of diluted ligation mixture (2 μ l of the ligation added to 8 μ l TE) in a pre-chilled 13 mm x 100 mm disposable glass test tube (Canlab) and incubated on ice for 30 minutes. The cells were then heat shocked for 90 seconds at 42°C without agitation. Two hundred μ l of exponentially growing JM109 cells were added to the competent cells along with 10 μ l of 100 mM IPTG, and 50 μ l of Xgal (2% w/v in dimethyl formamide). The cells were then overlayed onto B-plates (1% Bacto tryptone, 0.8% NaCl, 0.001% Vitamin B1) with 3 ml B-top agarose (0.6% agarose in B-broth) and incubated overnight at 37°C.

b) Genomic DNA Cloning in Lambda Phage Charon 4A

Limit Eco RI digested strain 1402 DNA (250 μ g in 500 μ l) was loaded onto a saline gradient (5% - 20% linear gradient produced using an ISCO gradient former) and centrifuged in a Beckman SW50.1 rotor for 3 hours at 45 000 r.p.m. at 20°C. Twenty fractions (230 μ l) were collected from the bottom of the tube and 10 μ l aliquots were analysed by gel electrophoresis. Fractions containing DNA predominantly in

the 6-9 kbp range were pooled, NH_4Ac extracted (1M final), and EtOH precipitated. One mg of limit Eco RI digested λ 4A vector (Blattner *et al.* 1977) was similarly fractionated (500 μg DNA/gradient) and analysed. Fractions containing the approximately 19.9 and 10.5 kbp fragments (the left and right ends of the 45.4 kbp vector respectively) were pooled, NH_4Ac extracted and EtOH precipitated. Removal of the two internal Eco RI fragments (about 6.6 and 7.8 kbp) allowed for substitution of 7.1-20.1 kbp of insert DNA. Ligations of the strain 1402 6-9 kbp saline fractions to the λ Ch4A ends were performed and recombinant phage were packaged (Hohn and Murray, 1977) and infected into E.coli strain K802 cells. Using this procedure, a library of approximately 3×10^4 independent clones were constructed. Plaques were pooled and titered. The titered library was reinfected into strain K802 cells, lifted (Benton and Davis, 1977) and probed using the prehybridization, hybridization, washing and exposure conditions described for the cascade hybridizations with Bam HI/Eco RI- excised radiolabelled Cas-2 prepared as discussed below.

c) Screening of Recombinant DNA Libraries

Transformed cells were lifted (Benton and Davis, 1977) onto Biodyne membranes and screened for induction-specific sequence by hybridization to ^{32}P -single stranded cDNA synthesized from maltose-induced poly A+ RNA. This probe

was enriched for maltose-specific sequence by a modified cascade hybridization procedure (Zimmerman et al. 1980, Seligy et al. 1983). To the single strand cDNA prepared for competitive hybridization (described in E)a), one-tenth volume of 10 M $\text{NH}_4\text{Acetate}$ was added, along with 100 μg total RNA from strain 1402 glucose grown cells (which had been heated for 5 minutes at 65°C and then cooled on ice for 5 minutes) and the sample was EtOH precipitated for 10 minutes at -80°C .

The precipitate was pelleted, dried and resuspended in 200 μl of an annealing buffer consisting of 70% deionized formamide, 0.4M NaCl, and 30 mM PIPES (pH 6.4) and incubated for 4 hours at 50°C . Prehybridization, hybridization and washing conditions were as follows: the sample was added to an appropriate volume (5 ml at 1.0×10^6 c.p.m./ml was used to screen 10 filters of 7 cm diameter each) of hybridization solution (6xSSC, 1x Denhardt's solution, 0.05% sodium pyrophosphate, 20 $\mu\text{g/ml}$ tRNA) and hybridized at 65°C for 40 hours to the M13 lifts (prehybridized at 65°C for 2 hours in 6xSSC, 0.05% sodium pyrophosphate, 100 $\mu\text{g/ml}$ sonicated denatured salmon sperm DNA). The filters were washed once for 2 minutes in hybridization buffer at 60°C , twice for 15 minutes in 2xSSC, 0.1% SDS at room temperature, three times for 10 minutes in 0.1xSSC, 0.1% SDS at 50°C , and once for 2 minutes in 6xSSC, 0.05% sodium pyrophosphate at room temperature. The filters were sealed in plastic bags to

maintain moisture and exposed to Kodak X-Omat AR film at -80°C.

Putative M13 positives underwent differential hybridization (St. John and Davis (1979). Single strand ("+" strand) DNA was prepared from plaque-pure clones (Messing 1983) and 1 µg aliquots were spotted (in duplicate) onto Biodyne membrane according to the manufacturer's protocol. The dot blots were prehybridized (as discussed) and then hybridized for 40 hours at 65°C to first strand cDNA synthesized from message of either glucose-grown or maltose-grown cells. The filters were washed under stringent conditions and exposed to X-ray film (see D)f)).

d) Subcloning Experiments using Various Vectors

The preparation of M13 mp18 and mp19 vector, ligation of vector to insert, and transformation of ligation mixture into E. coli strains JM101 or JM103 was essentially the same as the cloning of cDNA in M13 (see F)a)). A major difference was that only specific fragments of the genomic Scas-1 sequence were subcloned into M13 specifically for the purpose of DNA sequencing (see I)a)). Fragments and vectors were cut by combinations of 2 different enzymes to produce different termini used for forced cloning (see below).

Fragments subcloned into M13 were first separated by electrophoresis (see D)c)) and then recovered from the agarose by electroelution onto DEAE membranes (NA-45,

Schleicher and Schuell) according to the procedure of Dretzen et al., (1981). The membrane was appropriately cut to match different gel comb/slot widths. The membranes were presoaked for 2 hours in 2.5 M NaCl, rinsed in water several times, and then stored in 1 mM EDTA, pH 8.0 at 4°C. DEAE alignment with the DNA and confirmation of electroelution of the DNA into the membrane was observed by exposure of the prestained gel (with ethidium bromide at a final concentration of 0.5 µg/µl) to long wave-length UV. DNA was eluted from the membranes by soaking them in 300-500 µl of elution buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1.5 M NaCl) for 1 hour at 68°C. The eluted DNA was extracted twice with n-butanol to remove ethidium, once with phenol/chloroform/isoamyl alcohol (50:48:2) to remove any residual agarose, and then precipitated with 2.5 volumes EtOH. The precipitate was pelleted (13 000 r.p.m., 30 minutes) and dissolved in water at 10 ng/µl.

Forced cloning (Messing, 1983) involves the use of reverse oriented homologous restriction sites found in M13 mp18 and M13 mp19, and provides a means of obtaining strand-specific templates for hybridization and/or nucleotide sequencing. For example, the 2.0 kbp 3' Eco RI/Hind III genomic fragment of the 7.6 kbp Scas-1 sequence was reverse-oriented when inserted into similarly digested M13 mp18 and M13 mp19. Hybridization of ³²P-primer extended "+" strand insert to maltose-induced RNA tested positive in M13 mp19 while

testing negative in M13 mp18. The two recombinant phage were also tested for complementarity by use of the "C-test" (Messing, 1983). This procedure can also be used to excise inserts by use of either restriction endonucleases or single strand nucleases to eliminate the M13 DNA (Seligy *et al.*, 1986). Template DNA (250 ng) of various subclones were annealed to each other in 50 μ l containing (final): 0.1 M NaCl 6% deionized formamide, 250 μ M EDTA pH 8.0, 0.03% SDS, and 0.01% bromophenol blue. The reaction was incubated for 1 hour at 65°C, and the results were analysed by agarose gel electrophoresis (see D)c)).

Ligation of fragments into vectors pUC 18 and pUC 19 (Yanisch-Perron *et al.*, 1985) previously treated with BAP (see F)a)) involved mixing of the respective, appropriately restricted DNA (20 μ g/ml final) at a vector to insert molar ratio of 1:2, in 20 μ l final volume containing 0.1 units ϕ 4 DNA ligase (see F)a). Examples of inserts include the 5.8 kbp EcoRI/SalI and the 7.6 kbp EcoRI Cas-2 fragments. The ligation mixtures were transformed into either JM101 or JM103 as described in section F)a) with modifications. The growth medium used was SOB (2% Bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 10 mM MgCl₂, 2.5 mM KCl and 10 mM MgSO₄), and following heat shock, 800 μ l of SOC (SOB + 2 mM glucose) media was added to the cells. The cells were then incubated for 1 hour at 37°C and 275 r.p.m. before being divided into 100 μ l aliquots and plated on LA (LB + 35 μ g/ml

ampicillin) plates. The plates were allowed to dry at room temperature, and then incubated upside-down overnight at 37°C.

Ampicillin-resistant (plasmid-containing) colonies were screened for insert using the replica plating and colony hybridization procedure of Grunstein and Wallis (1979) and the appropriate probe. Several positively hybridizing colonies were picked and tested for the presence of insert by purification of plasmid using the mini-plasmid procedure (see D)a)) followed by restriction analysis (see D)c)).

G) Use of Synthetic Oligonucleotide Sequences

a) Synthesis and Purification

Deoxyoligonucleotide primers were synthesized by Dr. W.L. Sung using a Biosystems Gene Synthesizer (see Table 3). Primer sequences were predicted from DNA sequence using the dideoxy method as described (see I)a)). Primers were purified away from incomplete reaction products by electrophoretic separation on a 10% separating-TAE polyacrylamide gel followed by elution from the gel (Dretzen et al., 1981).

b) Primer Extension of cDNA and Genomic DNA

Template insert (whether cDNA or genomic DNA) in either M13 mp18 or M13 mp19 was primer extended using the procedure of Messing (1983) with modifications. Ten μ l of template (1 μ g) was combined with 4 μ l of the appropriate

sequencing primer (see Table 3) and 1.5 μ l H-buffer (10 mM Tris-HCl pH 7.5, 5 mM MgCl₂). The sample was gently vortexed and placed in a water bath initially at 68°C. The bath was allowed to slowly cool at room temperature until dropping below 40°C. The following was then added: 0.5 μ l of 200 mM DTT, 2 μ l of each of 1 mM dCTP, 1 mM dGTP, and 1 mM dTTP, 2.5 μ l of α^{32} P-dATP (25 μ Ci, 800 Ci/mmol), 1.5 μ l H-buffer, and 2 μ l (2 units) pol I (Klenow). The sample was incubated for 20 minutes at 37°C, supplemented with 2 μ l chase (1 mM dNTPs), and further incubated for 20 minutes. The primer-extended template could then be digested with an appropriate restriction endonuclease (see D)e)), separated by agarose gel electrophoresis (see D)c)), and recovered from the agarose gel by electroelution into DEAE-cellulose (see F)b)). Table 3 lists the universal and Scas-1-specific synthetic primers used for sequencing of Scas-1.

H) Preparation of Radioactive DNA Probes

a) Labelling by Nick Translation

Double-stranded DNA probes were radiolabelled by replacing pre-existing nucleotides with radioactive nucleotides (Rigby et al., 1977; Maniatis et al., 1982). One μ g of DNA was nick translated for 1 hour at 16°C in 100 μ l containing: 50 μ M dCTP, 50 μ M dGTP, 50 μ M dTTP, 50 μ Ci α^{32} P-dATP (800 Ci/mmol), 50 mM Tris-HCl pH 7.2, 10 mM MgSO₄, 100 μ M DTT, 50 μ g/ml BSA, 50 ng/ml DNase I, and 5

Table 3. Synthetic primers used in sequencing of Scas-1 gene.

Universal Synthetic Primers

15-MER	5' P TCCCAGTCAC GACGT _{OH} ³
17-MER	5' P GTAAAACGAC GGCCAGT _{OH} ³

Scas-1-Specific Synthetic Primers

<u>PROBE</u>	<u>SEQUENCE</u>
18.1	5' P TATGTAAGGA AATCAGACCT AATGTTGTT _{CoH} ³
19.1	5' P TAGTAGCTCA ATGTGAGGCG TATTAATGAT _{OH} ³
3A (cDNA/mp19)	5' P CTATATACCA TTTCTCATAC TCACTATAGA _{CoH} ³
3A'	5' P GCCTGAGTTA ATGATCATAT CGGATTGTTG _{GoH} ³
B/PV18-3	5' P GCGGAGTAGG ACAATTGTTC CAATTGTGG _{CoH} ³
E/PV18-3	5' P GGCTCTCATC ATTCTATCTG ATACCAGAGA T _{CoH} ³
H/PV18-3	5' P GAGATAACAT GTACCACATG CTTTGTCTTG _{CoH} ³
B/S3-19B	5' P GGTTACGGCT TGA CTGTCTC AGTATCTCT _{CoH} ³
B/S3-19B'	5' P CTTAGTGCAC GGTAGGGCTG GAGGGTGG _{OH} ³
B/S3-19B RP	5' P CATGATGCAA GATCCTATAA TTTCAATCAC _{GoH} ³
B/S3-19B' RP	5' P GATGGCACGT GATTCTATAA ATACTAAGAC _{OH} ³

units E. coli DNA polymerase I. The reaction was stopped by addition of 10 μ l of 0.5 M EDTA and 10 μ l of 20% SDS. One hundred μ g of denatured salmon sperm DNA was added as a carrier and the nucleic acids were precipitated in 10% TCA on ice for 20 minutes. The precipitate was pelleted (13 000 r.p.m., 5 minutes) and unincorporated nucleotides were decanted. TCA precipitation was repeated, followed by EtOH precipitation. The final pellet was dissolved in 200 μ l H₂O and denatured for 20 minutes at room temperature by addition of 100 μ l 1 M NaOH. One μ l was spotted onto a glass fibre filter (Whatman GF/C) and total Cerenkov c.p.m. were calculated. The sample was neutralized by addition of 100 μ l 1 M HCl and 200 μ l 1 M Tris-HCl pH 8.0 before being quickly added to hybridization buffer containing the appropriate DNA blotted filter.

b) Labelling by Fill-In (Klenow) Reaction

DNA markers (e.g. λ EcoRI/HindIII, pBR322 HinfI) were radiolabelled after restriction endonuclease digestion (see D)c)) by filling in recessed 3' termini using DNA pol I (Klenow) as outlined by Maniatis et al., (1982). A final volume of 25 μ l contained: 1 μ g restricted DNA, 50 μ M unlabelled dNTPs (as needed), 10 μ Ci α^{32} P-dATP (3000 Ci/mmol), 50 mM Tris-HCl pH 7.2, 10 mM MgSO₄, 100 μ M DTT, 50 μ g/ml BSA, and 1 unit Pol I (Klenow). The sample was

incubated at room temperature for 30 minutes, and the reaction was stopped by addition of 1 μ l of 0.5 M EDTA. The sample was extracted once with phenol/chloroform/isoamyl alcohol (50:48:2) and the DNA was separated from unincorporated nucleotides by centrifugation through 1 ml columns of STE-equilibrated Sephadex G-50 (Maniatus et al., 1982).

I) Nucleotide Sequencing

a) M13 Dideoxy Method for DNA Sequencing

DNA sequencing was performed using the Sanger chain termination procedure (Sanger et al., 1977), involving single strand phage M13 DNA (template), the universal 15-mer, 17-mer and Cas-2-specific synthetic oligonucleotide primers (see G)b)), pol I Klenow enzyme (Messing, 1983) and α^{32} P-dATP to detect primer extension products. Using this procedure, a DNA strand is synthesized by pol I Klenow in vitro using the M13 template. Synthesis is initiated at only one site on the template where primer DNA has annealed. Primer extension is terminated when a nucleotide analog is incorporated which cannot be elongated. These chain terminating analogs are 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs) which lack the 3'-OH group needed for chain extension. Addition of the proper concentration of one of the four ddNTPs with dNTPs will result in termination of the chain at each site where a ddNTP can be incorporated.

By use of four separate reactions, each with a different ddNTP, complete sequence information is obtained. A radiolabelled deoxynucleotide ($\alpha^{32}\text{P}$ -dATP) is also added to each synthesis and the chain lengths are visualized by autoradiography.

DNA sequencing by chain termination was also performed using modified bacteriophage T7 DNA polymerase (the "Sequenase" system described by Tabor and Richardson (1987) and distributed by USB). In this procedure, DNA synthesis is performed in two steps: a labelling step and the termination step (using ddNTPs). In a single reaction, primer is extended using limiting concentrations of dNTPs (including radiolabelled dATP). These randomly extended chains are then added to four separate chain-terminating reaction mixtures containing higher concentrations of dNTPs plus one of the ddNTPs.

Use of modified T7 DNA polymerase tends to give equal intensity at each correct nucleotide, which is a problem using pol I Klenow. The pol I Klenow sequencing procedure tends to be more efficient in reading shorter terminated sequences (less than 100 bases) than the T7 sequencing procedure, which favours longer terminated sequences. Therefore Scas-1 sequencing was performed using both enzymes and their respective procedures.

PAGE was performed using either S1 or S0 equipment manufactured by BRL. Upon completion of electrophoresis,

the front glass plate was removed and Whatman 3 MM paper was used as a support for the gels during drying in a BioRad 483 slab gel dryer. The gels were dried at 80°C and then exposed to X-ray film.

b) Use of Synthetic Primers

The synthetic primers listed in Table 3 were derived from sequence originally obtained using either the 15 mer or 17 mer universal primers on Scas-1 subcloned fragments. The synthetic primers were used to extend the number of bases read in a particular subcloned fragment by initiating extension at a specific site within the cloned sequence which approached the limit of resolved sequence. Identification of overlapping sequence confirmed specificity of the synthetic probe on the specific template.

J) Yeast Transformation

To study heterologous expression of the Scas-1 gene in Schiz. pombe, DNA containing this sequence was transformed into strain 2448 using the lithium acetate procedure described by Yarger et al. (1986). Log phase cells were grown in 100 ml YPD (2% bacto peptone, 1% yeast extract, 2% glucose) medium to a concentration of about 2×10^7 cells/ml. The cells were washed once in 5 ml TE buffer, and once in 5 ml lithium acetate buffer (TE + 0.1 M LiAc). The pelleted cells were then resuspended in 1 ml LiAc buffer and incubated for 1 hour at 30°C. The now competent cells could

be used immediately or stored at 4°C for up to 2 days. To 0.1 ml cells (resuspended if settled), 15 µl of DNA (1 µg plasmid + 45 µg denatured salmon sperm DNA carrier) were added. The mixture was incubated for 30 minutes at 30°C before 0.7 ml PEG (40% polyethylene glycol 3350 in LiAc buffer) was added, and the mixture was reincubated for 1 hour at 30°C. The reactions were heated to 42°C for 5 minutes, pelleted, washed twice with 0.5 ml TE buffer (to remove the PEG) and finally resuspended in 0.1 ml TE. The cells were then plated on selective plates (minimal medium with 4 µg/ml of the appropriate amino acid supplement) and incubated for 2-5 days at 30°C.

III. RESULTS

A) Growth and Substrate Requirements for Amylolytic Activity

In order to assess growth and substrate requirements for amylolytic activity in these studies, strain 1402 cells adapted to glucose were transferred to minimal medium supplemented with either 1% w/v glucose, maltose, or soluble starch. Samples were removed at 1, 2, 3, 4, 5, 6, 7, 8, and 24 hours to monitor substrate assimilation and glucoamylase activity. As shown in Figure 1, depending on whether cells were harvested during exponential or stationary growth phase, a significant difference in the growth pattern was observed. Cells harvested during exponential growth were able to complete a doubling using each substrate (Figure 1A, C, and E), although there were differences observed in their growth patterns. Only cells transferred to maltose media and starch media displayed lags in growth which lasted for approximately 1 and 2 hours respectively. However, growth in all three media was greatest between 3 and 4 hours after inoculation, and essentially complete in less than 8 hours.

Glucose grown cells harvested in stationary phase displayed virtually no growth within the 24 hour time course of the experiment (Figure 1B). However, even in the absence of replication, these cells utilized the carbohydrate present in the media more rapidly and to a greater extent than the respective cells harvested during exponential growth (Figure 1A).

Figure 1. . Analysis of Schwann, castellii strain 1402 growth, glucoamylase activity, and free glucose in the media. Cells were harvested in either exponential (A,C,E) or stationary (B,D,F) phase and then transferred and grown in minimal + 1% glucose (A,B), 1% maltose (C,D), or 1% soluble starch (E,F) media. Cell concentration ($\times 10^8$ cells/ml), $\circ-\circ$; free glucose (nmoles glucose/ml/min), $\bullet-\bullet$; glucoamylase activity (nmoles glucose released/ml/min), $\Delta-\Delta$.

Figures 1C and 1D indicate that expression of glucoamylase activity occurred in the maltose supplemented cells within the first hour. Levels of glucose production greater than 0.58 nmoles/ml/min were maintained from 3 through 8 hours, with peak rates of 0.66 and 0.62 nmoles glucose /ml/min at 3 and 4 hours in the exponential and stationary cultures, respectively. In contrast, glucoamylase activity was not detected in the soluble starch supplemented media until either the 5th (Figure 1E) or the 3rd (Figure 1F) hour following the shift from glucose media, and levels of activity greater than 0.50 nmoles glucose/ml/min were never observed at any time in either of the two cultures. The levels of glucoamylase activity in the glucose grown cultures had to be very low as no greater than 0.03 nmoles glucose released/ml/min could be detected. In all cases, glucoamylase activity was very low or absent in the 24 hour samples.

The levels of free glucose detected in the induced cultures at all times greatly exceeded the levels produced under assay conditions, indicating that the cells were not assimilating the glucose as quickly as it was being produced. As indicated in Figures 1C and 1D, glucose was detected in the maltose cultures within the first hour, and appeared to be greatest 5 hours after inoculation. By 7 hours, the amount of liberated glucose dropped to approximately one half the maximum and there after the

decline was gradual through to completion of the time course. Free glucose accumulation was considerably slower in soluble starch supplemented media, with detection of extracellular liberated glucose occurring at 5 hours (late log stage) in the growing culture and at 3 hours in the stationary culture. Free glucose rapidly declined in the culture which underwent growth; however it was far more gradual in the arrested culture. Under both culture conditions, appearance of glucoamylase activity was more rapid and the amount of glucose liberated was greater in maltose than in soluble starch.

Calleja et al. (1982, 1984) observed that in Schwann. alluvius continuously cultured in starch media, extracellular amylolytic activity (α -amylase and glucoamylase) was minimal when cells were dividing most actively, but increased tenfold at the end of exponential growth and was maximal in stationary phase. Because of this, they felt that there was a developmental role for the appearance of amylolytic activity in the sense that excretion occurred outside the mitotic cycle. Simoes-Mendes (1984) found that aerated cultures of Schwann. alluvius grown in starch media exhibited maximum glucoamylase production during exponential growth, and maximum α -amylase production at the end of exponential growth. In Figures 1A, C, and E, exponential growth was completed by the 4th hour of incubation and glucoamylase activity was detected from

early exponential through late stationary phase in maltose grown cells, and maximal activity was recorded during mid log growth. These results which have been repeated over several times indicate that amylolytic activity can occur at any stage of growth (not just post-mitotically) in order to adapt to changes in substrate.

B) Maltose as an Inducer of Glucoamylase in Schwann. castelii Strain 1402

Earlier studies (Sills et al. 1984a) on Schwann. castelii strain 1402 indicated that maltose was a better substrate for production of glucoamylase activity than soluble starch. Clementi et al. (1980) also found Schwann. castelii amylolytic activity to be slightly greater in cells grown in maltose than those grown in soluble starch. The α -amylase activity produced by strain 1402 in the presence of soluble starch was believed to be the result of very low, constitutive expression resulting in cleavage of starch to produce low molecular weight sugars such as maltose which could in turn pass through the cell membrane and induce synthesis of α -amylase (Sills et al. 1984b). Considering the relative speed at which free glucose and glucoamylase activity were produced by maltose grown cells, maltose was used as the substrate to further study induction of glucoamylase synthesis.

a) In Vivo Labelling of Total Proteins

In order to obtain a profile of newly synthesized proteins, in vivo labelling of total proteins with radioactive amino acids (see Materials and Methods) was performed on exponentially growing cells of strain 1402 shifted from glucose to maltose supplemented minimal medium. Cell-associated and extracellular samples were taken at various times (see Figure 2) and the total proteins were electrophoresed and compared to those from similarly labelled cells grown under non-inducing conditions.

As shown in Figures 2A and 2B, the cell-associated total protein profile from maltose-grown cells was similar to that of the glucose-grown profile, although production of several of these proteins appeared to be quantitatively different. In the maltose grown samples, bands at 138 kDa and 146 kDa were clearly present in the 30, 45, and 60 minute samples, (see arrows), but only faintly detectable in the 2, 3, and 4 hour samples. When total extracellular proteins were compared (see Figures 2C and 2D), two major bands measuring 78 kDa and 146 and several minor ones were observed in the maltose grown samples. In contrast, only trace amounts of two polypeptides measuring 66 kDa and 78 kDa were detected in the glucose grown samples, never reaching more than about 5% of the level in maltose samples. In the maltose grown samples, the 78 kDa band increased in intensity to a maximum at 2 to 3 hours, before diminishing.

Figure 2. In vivo labelling of total cell-associated and extracellular proteins from strain 1402. Yeast cells were grown in either minimal + 1% glucose (A,C), or minimal + 1% maltose (B,D) media in the presence of ^{35}S -methionine (10 $\mu\text{Ci/ml}$). Cells were harvested at various times and total protein from the supernatant cultures (C,D) and cell pellets (A,B) was subjected to PAGE and fluorography as described in Materials and Methods. Tracks (as indicated in A and B) a) 15 min; b) 30 min; c) 45 min; d) 60 min; e) 2 h; f) 3 h; g) 4 h; h) 5 h; i) 24 h; j) MW standards (top to bottom, 200, 92.5, 68, 43, 25.7). Arrows in B indicate (top to bottom) 146 kDa and 138 kDa proteins not observed in A or C. Arrows in D indicate (top to bottom) 146 kDa and 78 kDa proteins. Fluorographs were exposed for 4 days using Kodak X-Omat AR film at -80°C .

A

a b c d e f g h i j

B

a b c d e f g h i j

C

D

The 146 kDa band from the maltose grown cells was expressed at a low level in the 45 minute sample and then sharply increased at 4 and 5 hours, along with the minor bands of 120, 105, and 88 kDa. By 24 hours the 146 kDa protein band had almost entirely disappeared, with the appearance of trace bands of 53, 42, and 29 kDa.

b) Immunoprecipitation Studies

In order to determine which (if any) of the newly synthesized proteins were glucoamylase-related, the 45 minute cell-associated and the 4 hour extracellular samples grown under both conditions (i.e. glucose and maltose) were subjected to immunoprecipitation using anti-glucoamylase antibody (see Materials and Methods). As observed in Figure 3, four bands were immunoprecipitated from the maltose grown cell-associated sample (146, 138, 78, and 75 kDa). Only one band (78, kDa) was precipitated from the corresponding glucose-grown sample at less than 5% the level detected in the maltose grown sample. Two bands (146 and 78 kDa) were immunoprecipitated from the maltose-grown extracellular sample; only the 78 kDa band was precipitated from the corresponding culture supernatants of glucose-grown cells.

c) Western Blot Analysis

Similar analyses were carried out on non-labelled, total cell-associated and extracellular proteins by Western blotting and immunodetection using ^{125}I - protein A as the

Figure 3. Immunoprecipitation of in vivo labelled total proteins from strain 1402 grown under inducing and non-inducing conditions. Tracks:- a) maltose total cell-associated proteins harvested at 45 min; b) the immunoprecipitate of a; c) maltose total extracellular proteins harvested at 4h, d) immunoprecipitate of c; e) glucose total cell-associated proteins harvested from a 45 min culture grown on glucose; f) immunoprecipitate of e; g) glucose total extracellular at 4h; h) immunoprecipitate of g; i) MW standards: (top to bottom) 200, 92.5, 68, 43, 25.7, 18.3. Fluorographs were exposed for 4 days at -80°C . Arrows indicate Mr: (top to bottom) 146, 138, 78.

b c d e f g

radioactive probe. The results from these experiments are in general agreement with the previous immunoprecipitation experiments. The 138, and 146 kDa proteins were not observed in samples from glucose grown cells. Only faint, diffuse bands were detected (see Figure 4). Cell-associated and extracellular glucose grown samples taken after 2 hours showed a progressive accumulation of diffuse bands measuring approximately 48-52, 66, 80, 88 and 105 kDa (see Figures 4A and B). Similar sized bands were also detected as background in the maltose grown samples. As shown in Figure 5A, the cell-associated 78 kDa band appeared most strongly in maltose cells within the first 2 hours while the 146 kDa band peaked at 2 to 5 hours. The 78 kDa product also accumulated earlier in the extracellular samples (Figure 5B). The times at which the 78 and 146 kDa bands were detected were also the same as for the in vivo labelled bands (Table 4). The difference between the two immunoprobings techniques appears to be in the additional binding of bands in the Western blotting procedure. As shown in Figure 6, the binding of most of these bands, including the 78 kDa, can be eliminated by prebinding the antiglucoamylase antibody with a five-fold excess of total cell-associated protein from glucose cultures. The presence of lower MW bands in the glucoamylase standard suggests that these bands are degradation products of the glucoamylase protein. These results suggest that no detectable amounts

Figure 4. Analysis of cell-associated and extracellular glucoamylase by Western immunoblotting using ^{125}I -protein A. Presence of cell-associated (A) and extracellular (B) glucoamylase in total proteins from strain 1402 cells grown in minimal + 1% glucose. See Materials and Methods for details. Tracks: a) glucoamylase control; b) 15 min; c) 30 min; d) 45 min; e) 60 min; f) 2 h; g) 3 h; h) 4 h; i) 5 h; j) 24 h. Arrows in Panels A and B indicate Mr: (top to bottom) 105, 88, 78, 66, 42.

Figure 5. Analysis of cell-associated and extracellular glucoamylase by Western immunoblotting using ^{125}I -protein A. Presence of cell-associated (A) and extracellular (B) glucoamylase in total proteins from strain 1402 cells grown in minimal + 1% maltose. See Materials and Methods for details. Tracks: a) glucoamylase control; b) 15 min; c) 30 min; d) 45 min; e) 60 min; f) 2 h; g) 3 h; h) 4 h; i) 5 h; j) 24 h. Autoradiographs were exposed for 20 hours at -80°C . Arrows indicate Mr: Panel A (top to bottom) 146, 120, 105, 88, 78, 66; Panel B (top to bottom) 146, 120, 105, 78, 66, 59, 42.

a b c d e f g h i j

TABLE 4. Summary of glucoamylase-related products identified in the various preparations described in this study. Results are based on 10 separate experiments. Approximate sizes of the most abundant proteins as estimated by SDS PAGE (5% error) are given along with the earliest time at which the respective proteins were detected.

Source	Size (kDa)	Time (min)
Maltose Grown- Cell-Associated	146	30
	138	30
	78	15
	75	15
Maltose Grown-Extracellular	146	45
	78	30
Glucose Grown- Cell-Associated	78*	15
Glucose Grown-Extracellular	78*	30

* concentrations relative to maltose induced protein is approximately 5.6 to 6.6%.

Figure 6. Western/immunodetection of total proteins from strain 1402 cells grown under induced and non-induced conditions using glucoamylase antiserum competed with total cell-associated and extracellular proteins from 1402 cells grown in minimal + 1% glucose media. See Materials and Methods for details. Tracks: a) glucoamylase standard; b) maltose cell-associated, 3 h; c) maltose cell associated, 24 h; d) maltose extracellular, 3 h; e) maltose extracellular, 24 h; f) glucose cell-associated, 3 h; g) glucose cell-associated, 24 h; h) glucose extracellular, 3 h; i) glucose extracellular, 24 h; j) glucoamylase control. Refer to Figures 4 and 5 to compare with non-competed antibodies. Arrows indicate Mr: (top to bottom) 146, 120, 105, 96, 82, 78, 66, 60. Autoradiographs were exposed for 20 hours at -80°C.

of 146kDa glucoamylase are synthesized under glucose repressing conditions, while significant amounts of 78 kDa protein are constitutively produced.

The specificity of the anti-glucoamylase sera was also tested against in situ glucoamylase activity by combining supernatant from 4 hour maltose-induced cells (containing active extracellular glucoamylase) with various amounts of the antisera and then assaying for glucoamylase activity. Under the conditions described in the Materials and Methods, the anti-glucoamylase sera had no significant effect until a threshold concentration was reached (between 0.1 and 1 μ l anti-glucoamylase sera/ 30 μ l supernatant), whereby an approximately 25% reduction in activity occurred, and did not increase in the presence of ten fold more antisera. This experiment established that the anti-glucoamylase sera is specific for glucoamylase, although the reduction in activity is limited. The limited reduction in activity (as opposed to complete inactivation) implies that the antibody does not bind directly to the active site of the enzyme, but most likely recognizes a different region of the enzyme, causing either an alteration in the conformation of the enzyme or affecting access of substrate to the active site. In either case, the enzyme is only partially inactivated.

2

MicroD

d) Effect of Cell Concentrations on Maltose-Induced Glucoamylase Production

The maltose induction experiments were repeated under the conditions used to produce the results observed in Figure 5, except that competed antibody was used, and approximately 3×10^8 cells/ml were employed as the 1X inoculum. For these experiments, the analysis of cell-associated and extracellular samples was restricted to the first two hours, since induction was already shown to take place within this time frame. As shown in Figure 7, Western blot/immunodetection revealed that the production of glucoamylase was independent of cell concentration over a limited range. Analysis of the cell-associated samples (Figure 7A) revealed that by 30 minutes, the 138 and 146 kDa bands were detected at all cell concentrations. The relative amounts of these components appeared to increase linearly with cell concentrations up to 8X (2.4×10^9 cells/ml), while the 78 kDa protein had been effectively competed out and was not significantly detected. The amount of secondary products also appeared to increase linearly; the order of abundance of these products in the 2 hour sample was 66 > 105 > 88 > 78, 59, 53.

Extracellular products were not strongly detected in the first hour (Figure 7B), but appeared abundant in the 2 hour samples. Along with the 146 kDa protein, the secondary products detected by the glucoamylase antibody appeared to

Figure 7. Effect of cell concentration on maltose induced glucoamylase synthesis. Analysis of cell-associated (A) and extracellular (B) glucoamylase was carried out by immunoblotting of total protein using ^{125}I -protein A from strain 1402 cells grown in minimal + 1% maltose. The exponentially growing cells were inoculated at 1X (3.0×10^8 cells/ml), 2X (6.0×10^8 cells/ml), 4X (1.2×10^9 cells/ml), and 8X (2.4×10^9 cells/ml). Tracks: a) glucoamylase control; b) 1X, 15 min; c) 2X, 15 min; d) 4X, 15 min; e) 8X, 15 min; f) 1X, 30 min; g) 2X, 30 min; h) 4X, 30 min; i) 8X, 30 min; j) 1X, 45 min; k) 2X, 45 min; l) 4X, 45 min; m) 8X, 45 min; n) 1X, 60 min; o) 2X, 60 min; p) 4X, 60 min; q) 8X, 60 min; r) 1X, 120 min; s) 2X, 120 min; t) 4X, 120 min; u) 8X, 120 min. Arrows indicate Mr: Panel A (top to bottom) 146, 138, 120, 105, 88, 78, 66, 59, 53; and Panel B (top to bottom) 146, 105, 92, 88, 66, 59. Autoradiographs were exposed for 20 hours at -80°C

a b c d e f g h i j k l m n o p q r s t u

have increased substantially while the amount of 146 kDa protein detected had decreased. This indicated that breakdown of extracellular glucoamylase had exceeded synthesis. The extracellular pattern of breakdown (Figure 7B) differs from the cell-associated pattern (Figure 7A), since some products appear to be more represented than others.

e) In Vitro Translation of Glucoamylase mRNA

From the results already described it is clear that glucoamylase expression occurs very rapidly in strain 1402 cells when they are shifted from glucose to maltose-supplemented medium. It is also evident that the 146 kDa mature glucoamylase is found in the medium as well as associated with the cells. The 138 kDa glucoamylase is never detected extracellularly, but is found only in cell-associated samples. The information indicates that either the antibody recognizes two distinct glucoamylases, one 138 kDa and the other 146 kDa, or one glucoamylase of 146 kDa in size whose precursor is 138 kDa. As noted in the Materials and Methods (see B)a), a single 146 kDa active, column-purified glucoamylase was used to produce the anti-glucoamylase sera. Therefore, if the 138 kDa cell-associated protein is a separate glucoamylase species, it must carry similar epitopes or determinants as those of the 146 kDa polypeptide. Since glucoamylases are known to be

glycoproteins, and comprise anywhere from 3 to 30% carbohydrate. (Manjunath et al., 1983) it is possible that the 138 kDa protein is a deglycosylated or partially glycosylated intermediate. Measurement of the size of the in vitro translation product of glucoamylase messenger RNA isolated from maltose-induced cells could establish the size and number of nascent, non-processed glucoamylases produced. To do this, total RNA from maltose-induced and glucose treated cells was isolated and translated using a standard rabbit reticulocyte lysate which has not been supplemented with rough of KCl-washed microsomes (Jagus, 1987). Since protein was detected within the first hour, glucoamylase mRNA must have also been present within this time span.

Therefore, twenty µg aliquots of total RNA from the 60 minute glucose and maltose grown samples (and a 0 minute control) were in vitro translated using ³⁵S-methionine and rabbit reticulocyte lysate. For comparison, half of each of the products were fixed in Laemmli buffer (Laemmli, 1970) while the other half was immunoprecipitated using anti-glucoamylase sera before electrophoresis and fluorography. While similarities and differences existed between the glucose and maltose translation profiles shown in Figure 8, the most significant difference was the presence of an approximately 120 kDa polypeptide in the maltose-induced sample which was absent in the glucose sample. This 120 kDa polypeptide was the most abundant product synthesized from

Figure 8. Glucoamylase precursor identified by ^{35}S -methionine labelled in vitro translation of total RNA and immunoprecipitation with antiglucoamylase serum. Tracks: a) rabbit reticulocyte lysate without exogenous RNA (control), b) 0 min (minimal salts washed, pre-shifted control) total; c) glucose total, 60 min, d) immunoprecipitate of c, e) maltose total, 60 min, f) immunoprecipitate of e. Bands indicate MW standards (top to bottom): 200, 92.5, 68, 43, 25.7. Fluorograph was exposed for 3 days at -80°C .

the maltose-induced RNA, and it was also the only protein which was immunoprecipitated from either preparation. A slightly larger protein of about 130 kDa was produced in both the maltose and glucose samples, but it failed to precipitate with the glucoamylase antisera. The 130 kDa protein shown in Figure 8 of 60 minute maltose appears lower than that in the 60 minute glucose sample. However, this product is translated at a higher level in later stages of the maltose sample (see Figure 9)

The presence of the single immunoprecipitable 120 kDa glucoamylase synthesized in vitro indicates that there is only one nascent form and that it is further processed to a 138 kDa cell-associated glucoamylase and to the secreted, 146 kDa glucoamylase. It still remains to be determined if the 138 kDa polypeptide is a direct precursor of the 146 kDa protein due to two levels of glycosylation.

To further study the induction profile of the 120 kDa precursor glucoamylase in glucose and maltose-supplemented medium, a time course analysis was undertaken whereby cells grown in either glucose or maltose-supplemented minimal medium were harvested at 15, 30, 60, and 90 minutes and at 2, 3, 4, 5, and 24 hours. Total RNA was extracted from each of these samples and twenty ug aliquots were translated in vitro and the products electrophoresed and fluorographed as in Figure 8. The results of this experiment are shown in Figure 9. The immunoprecipitable 120 kDa protein described

Figure 9. Time course of ^{35}S -methionine labelled *in vitro* translations of total RNA isolated from cells grown in either glucose (A) or maltose (B) medium. Tracks: a) 15 min; b) 30 min; c) 45 min; d) 60 min; e) 2 h; f) 3 h; g) 4 h; h) 5 h; i) 24 h. Arrow in A indicates approximately 130 kDa protein. Arrows in B indicate (top to bottom) 130 and 120 kDa proteins. Bands indicate MW standards (top to bottom): 200, 92.5, 68, 43, 25.7. Fluorographs were exposed for 20 hours at -80°C .

in Figure 8 was clearly detected in the maltose-grown samples, but not in the glucose-grown samples. In the maltose grown samples, the 120 kDa nascent glucoamylase polypeptide was faintly present at 15 minutes and reached a maximum intensity by 60 minutes. The level of in vitro translated glucoamylase diminished by 3 and 4 hours, but appeared again at 5 hours. This observation may have been due to a transient build up of endogenous glucose to a threshold level which resulted in at least partial repression of transcription. When this level diminished, transcription may have again been induced, however this time to a lesser extent due to diminished maltose levels or possibly other limiting components. Differential levels of amylolytic activity over a time-course incubation have been previously described for yeasts such as Endomycopsis fibuligera, Pichia burtonii, S. diastaticus, Schwann. castellii, and Schwann. occidentalis (Sills and Stewart, 1982), however these previous analyses did not take place at the level of transcription. In vitro translation of RNA from 24 hour cells was found to be consistently poor, possibly due to partial breakdown of messenger RNA.

C) Glucose as a Repressor of Glucoamylase Production

a) Effect of Glucose on Induced Cells

Amylolytic activity in Schwann. castellii is repressed at glucose concentrations greater than 3 mM (Sills and Stewart,

1982). In these studies, it has been clearly demonstrated that glucoamylase synthesis is repressed in strain 1402 cells grown in minimal medium + 1% glucose (56 mM). Since cells were pregrown in minimal medium + 1% glucose, they were essentially adapted to growth in medium which did not require expression of glucoamylase. While it was demonstrated that uninduced cells could be rapidly induced to produce glucoamylase, the ability of glucose to repress induced cells (actively transcribing and translating glucoamylase) had not been tested.

Therefore, to test the ability of glucose to repress induced cells, the following experiment was performed. Cells pregrown in minimal medium + 1% glucose were shifted to minimal medium + 1% maltose. One set of samples (control) were harvested after 30, 60, 90, 120, 150, and 180 minutes. To a second set of samples, glucose (1% w/v) was added to the maltose-supplemented medium after 30 minutes incubation under inducing conditions, and cells were then harvested after 30, 60, 90, and 120 minutes growth in the presence of both sugars. These samples were analysed by Western immunoprobng of cell-associated and extracellular total proteins and in vitro translation of total RNA followed by immunoprecipitation of glucoamylase precursor product. Results of these experiments are shown in Figures 10-12.

The maltose control samples yielded similar results to those presented in Sections A) and B). Western

Figure 10. Effect of glucose on induced cells. Immunodetection of strain 1402 cell-associated proteins using Protoblot system. Tracks: a) 0 min (minimal salts washed, pre-shifted control); b) maltose, 30 min; c) maltose, 60 min; d) maltose, 90 min/glucose, 30 min; e) maltose, 120 min/glucose, 60 min; f) maltose, 150 min/glucose, 90 min; g) maltose, 180 min/glucose, 120 min; h) maltose, 90 min; i) maltose, 120 min; j) maltose, 150 min; k) maltose, 180 min. Arrows indicate M_r (top to bottom): 146, 138, 105. Bands indicate MW standards (top to bottom): 200, 92.5, 68, 43, 25.7.

a b c d e f g h i j k

Figure 11. Effect of glucose on induced cells. Immunodetection of strain 1402 extracellular proteins using Protoblot system. Tracks: a) glucoamylase control; b) 0 min (minimal salts washed, pre-shifted control); c) maltose, 30 min; d) maltose, 60 min; e) maltose, 90 min/glucose, 30 min; f) maltose, 120 min/glucose, 60 min; g) maltose, 150 min/glucose, 90 min; h) maltose, 180 min/glucose, 120 min; i) maltose, 90 min; j) maltose, 120 min; k) maltose, 150 min; l) maltose, 180 min.; m) MW standards (top to bottom): 200, 92.5, 68, 43, 25.7. Arrow indicates 146 kDa glucoamylase.

Figure 12. Effect of glucose on induced cells. Immunoprecipitation of total RNA samples translated in vitro. Tracks: a) 0 min (minimal salts washed, pre-shifted control); b) maltose, 30 min; c) maltose, 60 min; d) maltose, 90 min/glucose, 30 min; e) maltose, 120 min/glucose, 60 min; f) maltose, 150 min/glucose, 90 min; g) maltose, 180 min/glucose, 120 min; h) maltose, 90 min; i) maltose, 120 min; j) maltose, 150 min; k) maltose, 180 min. Arrow indicates 120 kDa preglucosylase. Bands indicate MW standards (top to bottom): 200, 92.5, 68, 43, 25.7.

immunoanalysis of cell-associated total proteins revealed the appearance of induced 138 kDa and 146 kDa glucoamylase after 30 minutes growth in maltose supplemented medium. These proteins remained strongly visible throughout the 180 minute incubation. The 146 kDa glucoamylase was faintly detected in the extracellular medium in the 30 minute sample and reached a maximum by 120 minutes. Immunoprecipitation of RNA translated in vitro from the maltose control samples resulted in isolation of the 120 kDa glucoamylase precursor which was most abundant in the 30 and 60 minute samples, and slightly less abundant in the 90 and 120 minute samples before decreasing to an almost negligible level by 180 minutes.

In the samples involving maltose and glucose, however, significant changes had occurred. Thirty minutes after addition of glucose to maltose-induced cells, the level of the 138 kDa cell-associated glucoamylase had diminished by approximately 80%, and in the 60, 90, and 120 minute samples this level had decreased to less than 5% the levels found in the respective control samples. The amount of 146 kDa glucoamylase released into the medium also decreased in the samples with glucose addition to less than 10% the levels seen in respective controls. Even more striking was the absence of immunoprecipitable 120 kDa glucoamylase from the in vitro translation of RNA from the glucose-supplemented samples. These results indicate that glucose acts as a very

potent repressor of glucoamylase expression. This repression takes place very quickly (within 30 minutes) and occurs at the level of transcription as indicated by the absence of in vitro translatable glucoamylase mRNA.

b) Effect of Glucose Depletion/Starvation and 2-Deoxyglucose

The effect of glucose depletion in cells over a gradual period of time (24 hours) was observed in Section A (Results). This time, experiments were performed to examine the affect of depletion of glucose (its sudden removal) on cells adapted to growth in glucose-supplemented medium. After pregrowth in minimal medium + 1% glucose, the cells were washed in minimal salts medium to remove exogenous glucose, and the cells were reinoculated into minimal medium without a carbohydrate supplement. Cells were harvested after 30, 60, and 90 minutes and samples were analysed (as outlined in C)a) of Results). The results are shown in Figures 13, 15, and 17.

Cell-associated 146 kDa glucoamylase was detected in equal abundance at all three time points (Figure 13). The 138 kDa protein was most abundant in the 30 minute sample (although accounting for no more than 5% of the 146 kDa protein detected in the same sample), after which it was barely detectable. Compared to glucose and maltose controls (Figures 13 and 14), the 146 kDa cell-associated glucoamylase levels were about 3 times greater than those of

Figure 13. Western immunoprobíng of strain 1402 cell-associated total proteins using Protoblot system. Cells were grown in minimal medium supplemented with 1% of the indicated sugars and /or other conditions described. Tracks: a) glucoamylase control; b) 0 min (minimal salts washed, pre-shifted control); c) no sugar, 30 min; d) no sugar, 60 min; e) no sugar, 90 min; f) maltose, 30 min; g) maltose, 60 min; h) maltose, 90 min; i) maltose heat-shocked from 30-60 min; j) soluble starch, 30 min; k) soluble starch, 60 min; l) soluble starch, 90 min; m) lactose, 30 min; n) lactose, 60 min; o) lactose, 90 min; p) galactose, 30 min; q) galactose, 60 min; r) galactose, 90 min; s) MW standards (top to bottom): 200, 94.7, 68, 43, 25.7. Arrows indicate Mr (top to bottom): 146, 138, 82, 78.

Figure 14. Western immunoprobings of strain 1402 cell-associated proteins using Protoblot system. Cells were grown in minimal medium supplemented with 1% of the indicated sugars and /or other conditions described. Tracks: a) glucoamylase control; b) xylose, 30 min; c) xylose, 60 min; d) xylose, 90 min; e) cellobiose, 30 min; f) cellobiose, 60 min; g) cellobiose, 90 min; h) glucose, 30 min; i) glucose, 60 min; j) glucose, 90 min; k) glucose + cAMP, 30 min; l) glucose + cAMP, 60 min; m) glucose + cAMP, 90 min; n) 2-deoxyglucose, 30 min; o) 2-deoxyglucose, 60 min; p) 2-deoxyglucose, 90 min; q) 2-deoxyglucose + cAMP, 30 min; r) 2-deoxyglucose + cAMP, 60 min; s) 2-deoxyglucose + cAMP, 90 min; t) MW standards (top to bottom): 200, 94.7, 68, 43, 25.7. Arrows indicate M_r (top to bottom): 146, 138, 82, 78.

abed e f g h i j k l m n o p q r s t

Figure 15. Western immunoprobining of strain 1402 extracellular proteins using Protoblot system. Cells were grown in minimal medium supplemented with 1% of the indicated sugars and /or other conditions described. Tracks: a) glucoamylase control; b) 0 min (minimal salts washed, pre-shifted control); c) no sugar, 30 min; d) no sugar, 60 min; e) no sugar, 90 min; f) maltose, 30 min; g) maltose, 60 min; h) maltose, 90 min; i) maltose heat-shocked from 30-60 min; j) soluble starch, 30 min; k) soluble starch, 60 min; l) soluble starch, 90 min; m) lactose, 30 min; n) lactose, 60 min; o) lactose, 90 min; p) galactose, 30 min; q) galactose, 60 min; r) galactose, 90 min; s) MW standards (top to bottom): 200, 94.7, 68, 43, 25.7. Arrows indicate M_r (top to bottom): 146, 78.

a b c d e f g h i j k l m n o p q r s

Figure 16. Western immunoprobings of strain 1402 extracellular proteins using Protoblot system. Cells were grown in minimal medium supplemented with 1% of the indicated sugars and /or other conditions described. Tracks: a) glucoamylase control; b) xylose, 30 min; c) xylose, 60 min; d) xylose, 90 min; e) cellobiose, 30 min; f) cellobiose, 60 min; g) cellobiose, 90 min; h) glucose, 30 min; i) glucose, 60 min; j) glucose, 90 min; k) glucose + cAMP, 30 min; l) glucose + cAMP, 60 min; m) glucose + cAMP, 90 min; n) 2-deoxyglucose, 30 min; o) 2-deoxyglucose, 60 min; p) 2-deoxyglucose, 90 min; q) 2-deoxyglucose + cAMP, 30 min; r) 2-deoxyglucose + cAMP, 60 min; s) 2-deoxyglucose + cAMP, 90 min; t) MW standards (top to bottom): 200, 94.7, 68, 43, 25.7. Arrows indicate M_r (top to bottom): 146, 78.

a b c d e f g h i j k l m n o p q r s t

Figure 17. Immunoprecipitation of strain 1402 total RNA translated in vitro. Cells were grown in minimal medium supplemented with 1% of the indicated sugars and /or other conditions described. Tracks: a) 0 min (minimal salts washed, pre-shifted control); b) no sugar, 30 min; c) no sugar, 60 min; d) no sugar, 90 min; e) maltose, 30 min; f) maltose, 60 min; g) maltose, 90 min; h) maltose heat-shocked from 30-60 min; i) soluble starch, 30 min; j) soluble starch, 60 min; k) soluble starch, 90 min; l) lactose, 30 min; m) lactose, 60 min; n) lactose, 90 min; o) galactose, 30 min; p) galactose, 60 min; q) galactose, 90 min. Bands indicate MW standards (top to bottom): 200, 94.7, 68, 43, 25.7. Arrows indicate Mr (top to bottom): 120, 88, 78, 48.

the glucose controls, but only 1/10th the amount observed in the maltose samples. Extracellular glucoamylase (Figure 15) was detected in the 60 and 90 minute samples at about 1/20th the amount observed in the respective maltose controls (Figure 15). The glucose controls produced no extracellular glucoamylase at any time point (Figure 16). Immunoprecipitation of RNA translated in vitro resulted in detection of only trace amounts (less than 5%) of 120 kDa protein in the 30 and 60 minute samples compared to maltose controls (Figure 17). No 120 kDa glucoamylase precursor was detected in the glucose controls (Figure 18), however immunoprecipitates were detected at 48 and 52 kDa. The identity of these bands is not presently known, and they were not detected in the immunoprecipitations of 60 minute glucose-grown sample shown in Figure 8 (track D) or in Figure 12 after maltose-induced cells were supplemented with glucose.

These results indicate that glucoamylase is produced within 30 minutes of removal of exogenous glucose from glucose-adapted cells. While the amount of glucoamylase detected in the absence of glucose substrate is far less than under maltose-inducing conditions, it is still significantly greater than levels detected under conditions of glucose repression.

As discussed in the introduction, the glucose analog 2-deoxyglucose is transported into, but believed not to be

Figure 18. Immunoprecipitation of strain 1402 total RNA translated. in vitro. Cells were grown in minimal medium supplemented with 1% of the indicated sugars and/or other conditions described. Tracks: a) xylose, 30 min; b) xylose, 60 min; c) xylose, 90 min; d) cellobiose, 30 min; e) cellobiose, 60 min; f) cellobiose, 90 min; g) glucose, 30 min; h) glucose, 60 min; i) glucose, 90 min; j) glucose + cAMP, 30 min; k) glucose + cAMP, 60 min; l) glucose + cAMP, 90 min; m) 2-deoxyglucose, 30 min; n) 2-deoxyglucose, 60 min; o) 2-deoxyglucose, 90 min; p) 2-deoxyglucose + cAMP, 30 min; q) 2-deoxyglucose + cAMP, 60 min; r) 2-deoxyglucose + cAMP, 90 min; s) maltose, 60 min (control). Bands indicate MW standards (top to bottom): 200, 94.7, 68, 43, 25.7. Arrows indicate Mr (top to bottom): 120, 52, 48.

metabolized by yeast (Zimmermann and Scheel, 1977; Afanas'eva et al., 1978; van Uden et al., 1980; Sills et al., 1984b). This analog has been used inconjunction with various forms of mutagenesis to produce mutants which when grown in the presence of glucose are derepressed for amyolytic activity i.e. they do not undergo carbon catabolite repression and hence produce amyolytic enzymes. Examples of derepressed mutants in Schwanniomyces spp. are also cited in the Introduction (see Section D).

In an attempt to answer the question of whether 2-deoxyglucose represses glucoamylase expression as much as glucose, or whether a low level of synthesis (such as that found with cells incubated in the absence of glucose) is maintained, cells adapted to minimal medium + 1% glucose were shifted to minimal medium containing with 1% (w/v) 2-deoxyglucose. Cells were harvested at 30, 60, and 90 minutes and analysed as described in Section C)a) of Results. The results of these analyses are shown in Figures 14, 16, and 18. In all cases, 2-deoxyglucose was as effective in repressing glucoamylase expression as glucose and could not be classed with cells which expressed glucoamylase at a low level when incubated in the absence of carbohydrate. It therefore appears that 2-deoxyglucose represses glucoamylase expression at the level of transcription and to the same extent as glucose.

c) Effect of Other Sugars

Apart from the carbohydrates already studied, four additional sugars were tested on glucose-adapted cells (washed of exogenous glucose) for glucoamylase expression. These four additional carbohydrates were: cellobiose, galactose, lactose, and xylase. Cellobiose is a disaccharide composed of two glucose units joined by a β -1,4 glucosidic linkage (and hence only differing from maltose by the glucosidic linkage). While the β -1,4 glucosidic bond is not recognized by glucoamylase, cellobiose might be recognized to some extent by the induction mechanism which recognizes maltose, or they might share the same induction mechanism equally. Galactose is an epimer of glucose, differing only in the configuration at carbon number 4. Apart from inducing the glucose-repressible Leloir pathway genes (documented for S. cerevisiae in Section C of the introduction) which convert galactose to glucose, there is no known relatedness to glucoamylase expression. However, since it would be supplying the cells with a fermentable carbohydrate, it would be interesting to note whether the basal level of glucoamylase synthesis would differ with the level found in cells devoid of any other exogenous carbohydrate. Since Schwann. castelii is the only Schwanniomyces spp. capable of assimilating lactose (and cannot ferment this disaccharide of glucose and galactose which is joined by β -1,4-galactosidic linkage), it was

compared to maltose and cellobiose, two disaccharides which are fermentable. Xylose, a five-carbon sugar assimilated by Schwanniomyces spp. was also tested. Finally, soluble starch was also added as a control.

The results are shown in Figures 13-18. Cell-associated 146 kDa glucoamylase levels from cells shifted to cellobiose, galactose, lactose, and xylose were 5-10 times greater than cells grown in glucose or 2-deoxyglucose, about twice as plentiful as cells grown in the absence of carbohydrate, and on the average about 5 times less than the respective maltose controls. However, the 138 kDa precursor levels accounted for no more than 5% of the amounts detected in the respective maltose controls. These levels were comparable to those from samples lacking exogenous sugar, but several fold greater than those from cells given glucose or 2-deoxyglucose. Low levels of extracellular 146 kDa glucoamylase were detected in the test samples. These levels were comparable to the samples incubated in the absence of exogenously added sugar, but significantly (approximately 10-20 times) less than the maltose controls. No extracellular 146 kDa glucoamylase was detected in cells grown in either glucose or 2-deoxyglucose. Comparison of the levels of immunoprecipitated 120 kDa precursor glucoamylase produced from in vitro translated RNA isolated from the respective samples and controls indicated the presence of no more than 5% of the amount produced from the

maltose-induced controls. As already discussed, no 120 kDa glucoamylase precursor was detected in the glucose-grown controls.

It is interesting to note that the soluble starch samples were comparable to the test samples at the 30 and 60 minute time points. It was only at the 90 minute time point that an increase in glucoamylase expression appeared to be proceeding. However, this increase was still significantly less than that of the maltose controls at any of the three time points. This observation is not surprising as it was shown in Section A of the Results that starch took longer to induce glucoamylase expression and that the level of expression was not as great as with cells induced by maltose.

The results of this study indicate that, in general, three levels of glucoamylase expression take place: an enhanced level which is rapidly induced by maltose; a low constitutive level (approximately 5% of the enhanced level) found in cells grown in the absence of exogenous glucose or maltose; and a catabolite repressed level found in cells grown in the presence of either glucose or its non-metabolized analog 2-deoxyglucose.

d) Effect of Heat

The shifting of yeast cells from their normal growth temperature (such as 25-30°C) to an elevated temperature

(such as 39° C) results in the repression of normal protein synthesis and the induction of constitutively synthesized heat-shock proteins. This heat-shock response has been well documented in eukaryotes including yeast such as Saccharomyces cerevisiae (Lindquist et al., 1982)). This heat-induced shift in transcription is accompanied by rapid degradation of many preëxisting messages (Lindquist, 1981). It has also been demonstrated that the pattern of heat-shock induction differs according to the carbon source used for growth (McAllister et al., 1979; Miller et al., 1979). Cells grown in a fermentable carbon source display a higher level of constitutive synthesis of heat-shock proteins than cells grown in a non-fermentable carbon source. As well, cells grown in a fermentable carbon source transiently maintain the heat-shock profile before the normal protein profile returns. The induction of heat-shock proteins is more dramatic, and once established, is maintained indefinitely in cells grown on a non-fermentable carbon source.

It has also been shown in S. cerevisiae that RNA processing of intervening sequences of some transcripts, that are not immediately degraded, is arrested during heat-shock (Lindquist, 1987). This results in the detection (by Northern analysis using probes specific for these transcripts) of larger transcripts than normally observed.

Prior to this work there were no known studies on heat-shock response in Schwann. spp., in general, and in particular on what affect heat-shock has on cells actively producing glucoamylase. At the level of enzyme activity, it has already been noted that the glucoamylase from Schwann. castelli strain 1402 is thermolabile (Sills et al., 1983a,b). Therefore, the effect of a 30 minute heat-shock on maltose-induced strain 1402 cells was studied. Cells induced in minimal medium + 1% maltose for 30 minutes at standard temperature (30°C) were shifted to 39°C for 30 minutes before harvesting. Status of glucoamylase production was analysed by Western immunoblotting of glucoamylase in cell-associated and extracellular in vivo translated proteins as well as by in vitro translation of total RNA coupled with immunoprecipitation of nascent glucoamylase using anti-glucoamylase serum. All of these analyses were compared to cells induced by maltose at standard temperature, with samples being removed at 30, 60, and 90 minutes. Particular emphasis was placed on comparison of the heat-shock samples with the maltose induced 60 minute samples since both sets of samples were harvested after 60 minutes growth in maltose. The results of these analyses are shown in Figures 13, 15, and 17.

The 30 minute heat-shock did not result in a significant difference in either the size or the abundance of the secreted 146 kDa glucoamylase (Figure 15). However, a

significant difference was observed in the cell-associated glucoamylase profile (Figure 13). While the amount of 146 kDa protein was similar to its 60 minute counterpart, the level of 138 kDa protein was almost negligible (less than 10%). However, no difference in the amount of breakdown product was observed. In vitro translation of total RNA (see Figure 19) revealed that heat-shock resulted in the induction of at least 5 proteins (with M_r of 100-103, 96-98, 85-87, 74-77, and 40-42) which were clearly visible compared to the control samples. Four of these are similar in size to heat-shock proteins in S. cerevisiae (McAlister et al., 1979; McAlister and Finkelstein, 1980). Only a very faint band (less than 5% of the 60 minute control sample) was detected at 120 kDa. To test whether or not the distinctive 120 kDa maltose induced in vitro translated glucoamylase had shifted in size due to possible effects of heat-shock on transcription, immunoprecipitations were performed. The results of the immunoprecipitations (Figure 17) clearly indicated that no immunodetectable products, apart from the 120 kDa polypeptide (which again amounted to no more than approximately 5% of the 60 minute control), were made as a result of the heat-shock.

The significant reduction in the amount of cell-associated 138 kDa protein is believed to be due to conversion of existing 138 kDa protein to 146 kDa product with little or no replenishment due to degradation of

Figure 19. Effect of heat shock on expression of glucoamylase and putative heat shock proteins by analysis of total RNA translated in vitro. Strain 1402 cells were grown in minimal medium supplemented with 1% maltose. Tracks: a) 30 min; b) 60 min; c) heat shocked from 30 to 60 min. Arrows indicate Mr (top to bottom): 100-103, 96-98, 85-87, 74-77, 40-42. Bands indicate MW standards (top to bottom): 200, 94.7, 68, 43, 25.7. Fluorograph was exposed for 20 hours at -80°C.

transcript. The observation that there is never an accumulation of 120 kDa precursor protein in vivo indicates that processing to the 138 kDa precursor is rapid. In S. cerevisiae, core oligosaccharides are added to nascent polypeptide (such as invertase) during its translocation across the membrane of the endoplasmic reticulum (Scheckman, 1982), indicating that glycosylation can take place rapidly enough to prevent detection of levels of non-glycosylated protein. In the study on the effect of glucose on induced cells (C)a)) and in this study, it was observed that repression of glucoamylase transcription (as monitored by in vitro translation and immunoprecipitation) appeared to be coupled to the turnover of 138 kDa cell-associated glucoamylase. This is in contrast to the 146 kDa cell-associated glucoamylase which does not appear to be turned over very rapidly. Since the 138 kDa precursor was not found extracellularly, this product must be processed further to produce the 146 kDa mature protein which is secreted into the medium at a rate slower than its synthesis.

These results clearly indicate that glucoamylase expression is severely retarded (90-95% reduction) by heat-shock treatment of cells, and that repression occurs at the level of transcription. These results also indicate that processing and secretion of glucoamylase are not inhibited by heat-shock. Finally, the in vitro translation profile of

heat-shock message resulted in the induction of certain proteins that are not glucoamylase-immunospecific and most likely represent a well-documented class of polypeptides commonly referred to as heat-shock proteins.

e.) Effect of cAMP

Adenosine 3',5'-monophosphate (cAMP) is produced from ATP by the action of adenylate cyclase. While the role of cAMP is well understood in prokaryotes such as E. coli, its role in eukaryotes such as yeast is still speculative. In E. coli, cAMP acts as a positive regulator for operons involved in the metabolism of sugars such as galactose, maltose, arabinose, sorbitol and lactose (reviewed in Freifelder, 1983). For example, in the lac operon, cAMP binds to a catabolite activator protein (CAP), altering its conformational structure so that it can bind to a specific site on the lac promoter. With cAMP/CAP bound to the promoter, and in the absence of repressor, induction of the lac operon can take place. Cyclic AMP concentrations rise if cells are starved for a carbon source, and drop if glucose is present. This is the result of repression of adenylate cyclase production by a metabolite of the glycolytic pathway. Hence, cAMP is a positive regulator in prokaryotic gene expression, playing an essential role in activating these catabolite-sensitive operons, while in turn being regulated by carbon catabolite repression.

In yeast, cAMP plays many roles (see review by Matsumoto et al., 1985). Levels of cAMP are controlled by adenylate cyclase and phosphodiesterase. Cyclic AMP attaches to a regulatory subunit of cAMP-dependent protein kinase, resulting in release of the catalytic subunit of this enzyme which phosphorylates cellular proteins with ATP. Studies with a variety of yeast mutants have revealed that cAMP-dependent phosphorylation is involved in the G1 phase of the life cycle, conjugation, and the post-meiotic stage of sporulation, however, inhibition of this phosphorylation is necessary to induce meiotic division. Since some yeast enzymes are regulated by phosphorylation-dephosphorylation (Krebs and Beavo, 1979), it was thought that cAMP might be involved in catabolite repression. The opinions regarding the role of cAMP in catabolite repression in yeast and fungi have been inconsistent (van Wijk and Konijin, 1971; Schlenderer and Dellweg, 1974; Mahler and Lin, 1978; Matsumoto et al., 1982, 1983; Eraso and Gancedo, 1984).

Afanas'eva et al. (1978) published studies on glucose catabolite repression of glucoamylase biosynthesis in the yeast Saccharomycopsis (Endomycopsis) fibuligera, and in these studies they found that under conditions of catabolite repression by either glucose (up to 2% w/v) or 2-deoxy glucose (upto 5 mM), the addition of cAMP (to 50 μ M) resulted in about 95% derepression of glucoamylase activity. To test the possibility of derepression of glucoamylase

expression by cAMP in Schwann. castellii, strain 1402 cells were grown in minimal medium + either 1% (w/v) glucose or 2-deoxyglucose supplemented with cAMP (50 μ M final). Cells were harvested after 30, 60 and 90 minutes growth and monitored for the presence of cell-associated and extracellular glucoamylase by Western immunodetection, and for an in vitro translated immunoprecipitable product. The results are shown in Figures 14, 16, and 18). The addition of 50 μ M cAMP had no observable effect on derepression of glucoamylase expression in strain 1402 cells grown in either glucose or 2-deoxy glucose supplemented minimal medium. As well, all samples appeared identical to growth in the respective media without cAMP. It therefore appears that, under the specific growth conditions used in this study, cAMP failed to derepress glucoamylase expression.

D) Cloning of Maltose-Inducible Transcripts

The results in Sections A, B, and C have established that glucoamylase transcription is considerably enhanced by maltose, and that maximal levels of this maltose-induced message occur within the first hour of exposure to maltose. Using this information, a gene whose transcript is maltose-inducible was cloned from Schwann. castelli strain 1402. Results will now be focused on experiments involved in the cloning and characterization of this gene.

a) cDNA and mRNA Production

Total RNA was extracted from glucose-adapted cells which were shifted to maltose supplemented medium. Cells were harvested after 15, 30, 45, and 60 minutes and total RNA and poly A+ RNA were purified from these samples described in the Materials and Methods. Double stranded cDNA was synthesized from the poly A+ RNA as also described in Materials and Methods.

b) Construction and Isolation of cDNA Clones

The double stranded, blunt ended cDNA, synthesized from maltose-induced poly A+ RNA was blunt-end ligated with Sma I digested, BAP treated M13mp19 as described in Materials and Methods. The ligation mixture was used to transfect JM109 cells, resulting in a cDNA library of approximately 3.2×10^4 independant clones. These clones were screened with ^{32}P -labelled first strand cDNA made from the same RNA and then hybridized to a 100 fold excess of total RNA isolated from similarly harvested glucose grown cells (as described in Materials and Methods). This "cascade hybridization" was carried out in order to eliminate or reduce sequences common to glucose and maltose growth. It was reasoned that cDNA made to RNA specific to, or abundant in cells exposed to maltose would not be competed by glucose RNA and therefore would remain available to bind to complementary cloned sequences in the M13 library.

Approximately 3 200 positives were detected in the 32 000 clones screened. However, only 50 of these (1.6% of the total number detected) hybridized strongly to the cascade hybridization probe. Secondary screening of these 50 clones using similarly prepared probe reduced the number of putative maltose-induced positives to twenty four. A tertiary round of similar hybridizations reduced the number of potential positives to seventeen. Plus-strand DNA was prepared from duplicate plaques of fifteen of the putative maltose-specific clones. In two other cases, three plaques were chosen from plaque-pure "positives" which appeared to bind probe weaker than other positives from the same isolate. One microgram of phage DNA from each of these preparations were spotted onto two Biodyne membranes. Each filter was probed with ^{32}P -labelled first strand cDNA from either glucose grown cells, or maltose grown cells. These results are shown in Figure 20. Only one clone, represented in triplicate as samples 2, 10, and 11, hybridized significantly to cDNA synthesized from maltose grown cells, but not to cDNA synthesized from glucose grown cells. This clone was designated Cas-2 cDNA and became the focus of all the analyses that followed. Rescreening of the cDNA library with ^{32}P -primer extended Cas-2 cDNA (see below) resulted in the isolation of only one other cDNA fragment which was designated Cas-3A.

Figure 20. Dot blots of "+" strand recombinant M13 mp19 clones probed with radiolabelled sscDNA from (A) glucose-grown and (B) maltose-grown strain 1402 cells. Note clones 2, 10, and 11 which hybridized to maltose sscDNA, but not to glucose sscDNA. Autoradiographs were exposed for 18 hours at -80°C.

A

1
7
13
19
25
31

c) Analysis of Specific cDNA Clones By Hybridization to Total RNA and to Chromosomal DNA

Complementary radiolabelled cDNA insert was prepared from the phage DNA of Cas-2 and Cas-3A clones by primer extension and excision from the M13mp19 DNA portion using Bam HI and Eco RI endonucleases. The excised fragments were small (100-200 bp). These fragments were recovered by electroelution using DEAE-cellulose to bind the DNA (see Materials and Methods). After release of ^{32}P -cDNA fragment recovered from the DEAE membrane, the probe was hybridized to total RNA isolated from strain 1402 cells shifted for 1 hour in either Minimal + 1% glucose or Minimal + 1% maltose supplemented media. The RNA was electrophoresed in 1.0% agarose before blotting to nitrocellulose (Northern blot) and hybridization. The results of these analyses are shown in Figure 21.

Both Cas-2 and Cas-3A cDNA failed to hybridize to transcript in the total RNA from the glucose grown cells, however, significant binding to RNA in the total RNA of the maltose grown cells occurred. The RNA identified by both clones was the same, approximately 21s in size according other known RNA reference markers. The 21s RNA corresponds to about 3.15-3.25 kb in size. Taking into account about 150-200 nucleotides for 5' and 3' untranslated regions of the mRNA, the coding sequence would be sufficient for translation of a polypeptide of about 1000 amino acids.

Figure 21. A. Ethidium bromide stained total RNA isolated from strain 1402 cells harvested after growth for 1 hour in either glucose or maltose medium and electrophoresed in an agarose, formaldehyde gel. B and C. Duplicate samples probed with either radiolabelled Cas-2 (B) or Cas-3A (C) cDNA. Arrow indicates 21s RNA. Autoradiographs were exposed for 2 days at -80°C.

This number of amino acids would yield a polypeptide of about 110 to 120 kDa. This is the approximate size of the putative glucoamylase nascent polypeptide identified in the same RNA samples by in vitro translation.

To carry out chromosomal (genomic) DNA analysis, single limit restriction endonuclease digests were performed on strain 1402 genomic DNA using the restriction endonucleases Bam HI, and Eco RI. These digests (as well as others) were electrophoresed in a 0.7% agarose Tris-acetate (TAE) gel and blotted onto Biodyne nylon membranes. These blots were hybridized to similarly radiolabelled Cas-2 and Cas-3A cDNA sequences. Autoradiography of the washed filters after hybridization revealed that both Cas-2 and Cas-3A cDNA probed a single, approximately 7.6 kbp Eco RI genomic fragment, while only faintly recognizing 16.5 kbp sequence from the Bam HI digest. The relatively weaker binding of these probes to the Bam HI digest may have reflected less efficient transfer of the higher molecular weight fragment onto the Biodyne membrane. The 7.6 kbp Eco RI fragment was chosen for further analysis.

d) Nucleotide Sequence of Cas-2 and Cas-3A cDNA

The DNA sequence of Cas-2 and Cas-3A cDNA clones was determined as described in Materials and Methods. Sequencing of Cas-2 revealed that the cDNA insert in this clone was 108 bases in length, while the insert of Cas-3A

was slightly larger (144 bases). The Cas-2 sequence was found in the Cas-3A sequence shown in Figure 22. Further analyses requiring cDNA probe was done using Cas-3A because of its larger size.

e) Cloning of Genomic Sequences Complementary to Cas-2/3A cDNA

DNA from strain 1402 was limit digested with Eco RI and after fractionation by agarose gel electrophoresis, the size class corresponding to 7.6 (6 to 9) kbp was excised out and eluted for cloning. A library of these 6-9 kbp DNA was constructed using the bacteriophage Lambda Charon 4A as vector (see Materials and Methods). Approximately 3.0×10^4 independant clones were obtained. These clones were pooled, and about 1.0×10^5 plaques were screened with radiolabelled Cas-3A under stringent hybridization conditions. This procedure lead to the detection of about 3×10^3 putative positives. Thirty of these putative positives were picked and a secondary screening lead to eight positive clones. DNA from these positives digested with Eco RI each produced a single insert approximately 7.6 kbp in size. By Southern analysis, Cas-3A cDNA was shown to bind specifically to this 7.6 kbp insert. One of these clones was used for further analysis and was designated λ Ch4A.Scas-1.

Cas-2 (108 bases)

5' P TTTTATGTCTTTGTAATTAATAAAGGAAAAATTTATATATGTTTGTAT
AGTCTATATGAGTATGAGAAATGGTATATATTTATTGAGAAAAAAAAAAAAA_{OH} 3'

Cas-3A (144 bases).

5' P GCTGAATATTGTCCAGATGATAAATAATGAAGTTTTTTATGTCTTTGTAATTA
ATAAAGGAAAAATTTATATATGTTTGTATAGTCTATATGAGTATGAGAAATG
GTATATATTTATTGAGAAAAAAAAAAAAA_{OH} 3'

Figure 22. DNA sequence of cDNA clones Cas-2 and Cas-3A.

E) Characterization of the Scas-1 Gene

a) Restriction Endonuclease Mapping

Southern analysis of restriction endonuclease digests of λ Ch4A.Scas-1 probed with radiolabelled Cas-3A revealed that the 3' cDNA sequence bound to a 2.0 kbp Eco RI/Hind III fragment which was located at one end of the 7.6 kbp sequence. This 2.0 kbp fragment was subcloned into M13mp18 and M13mp19. When screened with 32 P-primer extended Cas-3A template, only the mp19 subclones bound to the probe. DNA sequencing of Cas-3A revealed its orientation in M13 mp19 which, combined with knowledge of its location on the 7.6 kbp fragment, indicated that the 21s Cas-2/3A transcript should be coded in its entirety within the 7.6 kbp Eco RI sequence.

Southern analysis of single and double restriction endonuclease digests of strain 1402 genomic DNA was further performed using 32 P-nick translated (Rigby *et al.* 1977) 7.6 kbp sequence purified from the λ Ch4A clone using DEAE-cellulose. The restriction map derived from this information is shown in Figure 23.

b) Organization of Scas-1 Gene and Flanking Sequence.

The physical maps showing the position of the Scas-1 gene with respect to various restriction endonuclease recognition sites defined by hybridization to chromosomal DNA (Figure 23) and to cloned DNA (Figure 24) are in good agreement.

Figure 23. Southern analysis of single and double restriction endonuclease digests of strain 1402 genomic DNA probed with radiolabelled 7.6 kbp Eco RI fragment containing Scas-1 gene. A. Ethidium bromide stained agarose-TAE gel after electrophoresis of digested DNA samples. B. Autoradiograph of probed Southern blot of A. Tracks: a) Hind III; b) Bam HI; c) Eco RI; d) Hind III; e) Kpn I; f) Pst I; g) Pvu II; h) Sal I; i) Xba I; j) Bam HI/Eco RI; k) Eco RI/Hind III; l) Eco RI/KpnI; m) Eco RI/PstI; n) Eco RI/Pvu II; o) Eco RI/Sal I; p) Eco RI/Xba I. C. Structural map derived from B. Arrow indicates region containing Scas-1 gene. Symbols: B=Bam HI; E=Eco RI; H=Hind III; P=Pvu II; S=Sal I; X=Xba. Autoradiograph was exposed for 2 days at -80°C.

U

Figure 24. Physical map of Scas-1. Map shows location of Scas-1 within the 7.6 kbp Eco RI chromosomal DNA fragment. Arrows below the fragment indicate location of various synthetic oligonucleotide primers used to sequence the 3 985 nucleotides defined by arrow above the map. The putative transcription unit begins with the promoter (TATA) and ends at the site of polyadenylation of 21s mRNA (polyA), defined by nucleotide sequence of cDNA clones 2-3A (see Figure 22 for actual sequence). Location of putative translation start and stop signals, coding regions (exons, E1, 2 and 3), intervening sequences (IVS-1, 2) and restriction endonuclease recognition sites along the 7.6 kbp Eco RI fragment are also indicated.

This indicates that no apparent DNA rearrangement has taken place by the cloning method.

As discussed, orientation of the Scas-1 transcription unit along the 7.6 kbp Eco RI cloned fragment was determined initially by use of the cDNA clones Cas-2 and Cas-3A. Sequences beyond the extreme upstream Xba I site do not probe the 21s maltose inducible RNA. In the absence of available amino sequence information to help define the Scas-1 gene, accurate nucleotide sequence was obtained by use of various primers in conjunction with subclones in M13 mp18 and mp19, defined by the various restriction sites indicated. M13 primers were used to obtain initial sequence that allowed for synthesis of other oligonucleotide primers specific to Scas-1. This method of continuous, "walking" along the sequence in both directions ensured the necessary overlap and repetition required for prediction of gene organization.

Definition of Scas-1 gene organization was computer assisted, making use of the knowledge of the transcript and consensus nucleotide sequences previously found to define transcription and translation "start" and "stop" sequences in yeasts and fungi (Gannon et al., 1979; Breathnach and Chambon, 1981; Zaret and Sherman, 1982). As expected from empirical tests, the 3 985 nucleotide sequence shown in Figure 25 contains very few "six base" endonuclease recognition sites. Search for open reading frames, in

either 3' or 5' direction in all six phases revealed two blocks, a 5' block of 176 codons and 773 codons downstream of the first Xba I site. The latter is consistent with the largest RNA protected fragment (about 2 500 nucleotides) after treatment with the single strand specific S1 nuclease. The gap between these two coding segments was closed by hypothesizing two introns, flanking a smaller 77 codon block. Combined the total coding region could produce a polypeptide between 113.4 kDa and 118.6 kDa in size.

The sizes of the two hypothesized introns and the three exons are consistent with those reported for other yeast and fungal genes (Gines, 1987). The prediction of the location of the two 5' exon/intron and the two 3' intron/exon junctions was made using a consensus sequence formula derived from work on the eight introns found in each of the two taka-amylase genes of the ascomycetous fungus, A. oryzae (Seligy, Dove and Gines, unpublished), along with published information on other yeast and fungi (Yoshizumi and Ashikari, 1987). The boundaries of these intervening sequences correlate well with the presence and position of an internal conserved sequence (ICS), AAGTACT found within each intron. Search for the S. cerevisiae consensus sequence indicated that this sequence was not present. Therefore it would not be surprising to find that the Scas-1 may not be expressed in this yeast. These predictions permit future testing using RNA in conjunction with electron

microscopy and S1 nuclease (Seligy, Dove, Luck, and Dowhanick in progress).

Analysis of regulated transcription of a few yeast and fungal genes so far indicate that nucleotide sequences immediately flanking the promoter are characterized by clustering of inverted and direct repeating sequences (Gines, 1987). As shown in Figure 25 there is a cluster of both types of repeating sequences immediately upstream of the putative Scas-1 transcription promoter and also immediately downstream of the site of polyadenylation, predicted from cDNA cloning. The presence and positioning of these sequences further strengthened the proposed location of the transcription unit of this gene. Given existing techniques for introducing site-directed changes, the role of these sequences in regulated expression of Scas-1 by glucose and maltose can now be studied.

c) Scas-1 Gene Hybridization to Total RNA

The 7.6 kbp Eco RI fragment and 5.8 kbp Sal I/Eco RI derivative sequence containing Scas-1 were ³²P-nick translated and hybridized to a duplicate of the Northern blot used in Figure 21. The result was identical to that observed with Cas-2/3A cDNA clones. No significant binding occurred to transcripts in the glucose-grown cells, while strong binding to a 21s RNA in maltose-grown cells was observed. This indicates that under the conditions of

Figure 25. Nucleotide sequence of Scas-1 gene. Nucleotide sequence was obtained by dideoxychain termination (see Methods), using the oligonucleotide sequences indicated in Figure 24 and Table .3 as primers for DNA polymerase. Location of the putative transcription unit including "CAAT", "TATAA", start (ATG), STOP (TAG), coding regions (exons, E1, 2, 3), intervening sequences (IVS-1, 2), reference endonuclease restriction sites, and direct (DR 1-3) and indirect (IR 5) repeats of nucleotides greater than 8 in length are all noted.

+1 CAGGCTATCAGTGTAGTTGACCTAAAAACATCTACTAGTTTGAAGAAAGAAGTAATAGCTTTAGAAGAATATTTTGTACAGATATATGTCACATTAAAGTGTATTGAATATA
 +60
 +120 AGTGCATTATTGTTTGTAAATATCTCTCTGACGCGAGCTTATGATGTACAAATACATAGAACTACTATGAAGACTTCTCAAAATCTCTCTCTGATATAGCTAGCGAAGGGTCTACTAAT
 DR1 → TATA → DR2 → IR1 → CAT → IR2 → CAT → IR2 → DR1 → XbaI+355
 +240 CCAACGAAGATATAAAAAATGGAATTCATCTTTAAGAAATGTTTCGGTCTCTCTATAAAGANGTGACAAGAAATATGCACCTTTAAGCAATGATCAATTAAATCGTCAACATATTCTAG

5 10 15 20 25 30
 ATG ATA ATA TAC ATA ACT TCA AAT TCA TTA TCA CAA TTT TTG TGG TAC AAC AAA TAC TCA AGA CCA TAC AAA GCA CTG AAT GAG CAT TCT
 met ILE ILE TYR ILE THR SER ASN SER LEU SER GLN PHE LEU TRP TYR ASN LYS TYR SER ARG PRO TYR LYS ALA LEU ASN GLU HIS SER
E1

35 40 45 50 55 60
 ACT TTA TTT AAC CAA ATA ACG CAC TGC AAT CCG GAG TTC GAA TTT GGA ACA AAG ATA GAG GGC GCA GGG CGC TGT GTT GAG AAC ATA GCG
 THR LEU PHE ASN GLN ILE THR HIS CYS ASN PRO GLU PHE GLU PHE GLY THR LYS ILE GLU GLY ALA GLY ARG CYS VAL GLU ASN ILE ALA

65 70 75 80 85 90
 AGG GTG AAA ATA CCC AGC ATG AAG AGA CTA CAC CTA AAC AGC ACT GTG CAT GAG AGA AGA CCG AAA TCA ATG ACA ACG AAG GTA ATG ACA
 ARG VAL LYS ILE PRO SER met LYS ARG LEU HIS LEU ASN SER THR VAL HIS GLU ARG ARG PRO LYS SER met THR THR LYS VAL met THR

95 100 105 110 115 120
 AGA CTA AAG GAG CCA ATA TCC AAA AAC CTA GAC TAT AGA TCA CCT CAA GAG TAT CAA AAA TTA GCG GCC AAA CAA CAG AAA AAT TAT GCT
 ARG LEU LYS GLU PRO ILE SER LYS ASN LEU ASP TYR ARG SER PRO GLN GLU TYR GLN LYS LEU ALA ALA LYS GLN GLN LYS ASN TYR ALA

125 130 135 140 145 150
 GAG CTA TGC CCA GGC TAC AGC AAG AGA ATC TAC AGT GTT GGA TAT ATA GGT AGA CAT GCG CAA GGC TAC ACG CTA AGC AAG GTC ACA AGA
 GLU LEU CYS PRO GLY TYR SER LYS ARG ILE TYR SER VAL GLY TYR ILE GLY ARG HIS ALA GLN GLY TYR THR LEU SER LYS VAL THR ARG

155 160 165 170 175 180
 GGC ACC ACC CTC CAG CCC TAC CTG CGA CTA AGA TAT TTT ATG ATT CTG ATA ACA CGT GTC ATA TCC TAC AAG CGA CAC TA GTAACTGGCTA
 GLY THR THR LEU GLN PRO TYR LEU ARG LEU ARG TYR PHE met ILE LEU ILE THR ARG VAL ILE SER TYR ASN ARG HIS TY

185 190
 AGTCTTATCGAATGTCTCTAAGTACTTACGAACGGTCGATTAACTAC C GAT TGG ATT ACA CAA AAT GAT TTG AGA AAA AAG AAA ATA CAC GTG AAG TTA
 IVS-1 R ASP TRP ILE THR GLN ASN ASP LEU ARG LYS LYS LYS ILE HIS VAL LYS LEU

E2

195 200 205 210 215 220
 TAC CCC TCA ATC AGA GTT AAT TAT GGC AGA AAA AAC AAA CAA TAT CTT TTA ACA AAG TCC GTG ATA TGC ATT GAT ACA TTG GCT GAC CCG
 TYR PRO SER ILE ARG VAL ASN TYR GLY ARG LYS ASN LYS GLN TYR LEU LEU THR LYS SER VAL ILE CYS ILE ASP THR LEU ALA ASP PRO

225 230 235 240 245 250
 TAC GTG GCG GCT ATA GTC CTC ATC CTA CTG AAA CAC CCT GTA GTG GGT ATA TCC AAG AAA ACA TCT GAG CTT CAA AAC TAT AAT CAA CCC
 TYR VAL ALA ALA ILE VAL LEU ILE LEU LEU LYS HIS PRO VAL VAL GLY ILE SER LYS LYS THR SER GLU LEU GLN ASN TYR ASN GLN PRO

255 260
 TAT GAT T GTATTATAATGTGCTAGAAAGCTGTAACACATAGCTCTAGAATATTGTTCAAGTACTAAGCTTCTAGGATATTAAAG TT AGT GCT TCG TTG AAT GGT CTG
 TYR ASP P HE SER ALA SER LEU ASN GLY LEU

265 270 275 280 285 290
 ATC TTT TCT AAG GTA ACA CGC CCC CTC CTT TTT CTC TAC CAG TTG AGA GAT ACT GAG ACA GTC AAG CCT GAA CCA AAC GAT CTT AGA TGT
 ILE PHE SER LYS VAL THR ARG PRO LEU LEU PHE LEU TYR GLN LEU ARG ASP THR GLU THR VAL LYS PRO GLU PRO ASN ASP LEU ARG CYS

295 300 305 310 315 320
 GAT TAC ATA ATT ACT TCC TCG GTT TTT TTT TTC ATT AAA TTT GCC TCG GTA CAC GAG CAT ATG AAT TTG TGG AGA ATG ACA TAT ATA TAT
 ASP TYR ILE ILE THR SER SER VAL PHE PHE PHE ILE LYS PHE ALA SER VAL HIS GLU HIS met ASN LEU TRP ARG met THR TYR ILE TYR

E3

325 330 335 340 345 350
 ATA TAT AGT ATT AGC ATC TTA TCT AAA GTA TTT GGT TTC TGC ATA TTT GAA ACA CAA ATA AAG ACA ACA TTT TTC ATT ATG GTT TAT GGT
 ILE TYR SER ILE SER ILE LEU SER LYS VAL PHE GLY PHE CYS ILE PHE GLU THR GLN ILE LYS THR THR PHE PHE ILE met VAL TYR ALA

355 360 365 370 375 380
 TCA AAT TTT ACT AAC TCA TCA AAT CCC CCA AAT CCA AGA GAA TCT ATT CAA AAG GAA AGA GAT GCA GCT AAA TTT TCT CCT GTG GAT ATG
 SER ASN PHE THR ASN SER SER ASN PRO PRO ASN PRO ARG GLU SER ILE GLN LYS GLU ARG ASP ALA ALA LYS PHE ASN PRO VAL ASP met

385 390 395 400 405 BamHI 410
 CAT GTT TTT TTA GAA GGA TCA AAA GAA AGA TCT GAA TCA TTT AAA ACG TTG GTT CAA CAA ATG GAA AGG GAT CCT ATT TTA GCA TCC AGT
 HIS VAL PHE LEU GLU GLY SER LYS GLU ARG SER GLU SER PHE LYS THR LEU VAL GLN GLN met GLU ARG ASP PRO ILE LEU ALA SER SER

415 420 425 430 435 440
 CCT AAA GAC TAC GAT TTG ACA AAA GAC CAA CAA AGA GAA TTG ACG GCA ATA AAA ATC AAC CGT TTA AGC AGA TAT CTT GAA ACC GAA GGC
 PRO LYS ASP TYR ASP LEU THR LYS ASP GLN GLN ARG GLU LEU THR ALA ILE LYS ILE ASN ARG LEU SER ARG TYR LEU GLU THR GLU GLY

445 450 455 460 465 470
 TCC TTT CAA GAA TTT AAC AGA AGA TTG TCG TTG GTC GGG GTA TTT GAC CCA CAA GTA GCC ACA AGA ATA GGT ATC AAC TTA GGG TTG TTC
 SER PHE GLN GLU PHE ASN ARG ARG LEU SER LEU VAL GLY VAL PHE ASP PRO GLN VAL ALA THR ARG ILE GLY ILE ASN LEU GLY LEU PHE

475 480 485 490 495 500
 TTG AGC TGT ATA AGA GGT AAT GGT ACT GCC GAT CAA CTT CAA TTC TGG GCC ACA TAC AAA GAA ACA TGC TTC GTT AAG CAT ATG TAC GGA
 LEU SER CYS ILE ARG GLY ASN GLY THR ALA ASP GLN LEU GLN PHE TRP ALA THR TYR LYS GLU THR CYS PHE LEU LYS HIS met TYR GLY

505 510 515 520 525 530
 TGC TTC GGT ATG ACT GAG TTG GCA CAT GGT TCT AAT GTC ATG GGA TTG GAA ACC ACA GCG ACT TTT GAT AAA GAG ACA GAC GAG TTT ATC
 CYS PHE GLY met THR GLU LEU ALA HIS GLY SER ASN VAL met GLY LEU GLU THR THR ALA THR PHE ASP LYS GLU THR ASP GLU PHE ILE

535 540 545 550 555 560
 ATT AAT ACG CCT CAC ATT GGA GGT ACT AAA TGG TGG ATC GGT GGT GCT GCA CAT TCA GCT ACA CAC TGT ACC GTT TAT GCT AGA TTA ATT
 ILE ASN THR PRO HIS ILE GLY ALA THR LYS TRP TRP ILE GLY GLY ALA ALA HIS SER ALA THR HIS CYS THR VAL TYR ALA ARG LEU ILE

565 570 575 580 585 590
 GGT GAT GGA GAA GAC TAT GGT GTT AAA ACA TTT GTT GTA CCA TTG AGA GAC TCA AAT CAT GAA TTG AAT CCA GGT GTC GCT GTA GGA GAT
 VAL ASP GLY GLU ASP TYR GLY VAL LYS THR PHE VAL VAL PRO LEU ARG ASP SER ASN HIS GLU LEU ASN PRO GLY VAL ALA VAL GLY ASP

595 600 605 610 615 620
 ATT GGT GCT AAG ATG GGT AGA GAT GGT ATC GAT AAT GGT TGG ATT CAA TTT TCG AAT GTT AGA ATC CCA AGA TTC TTC ATG TTA CAG AAA
 ILE GLY ALA LYS met GLY ARG ASP GLY ILE ASP ASN GLY TRP ILE GLN PHE SER ASN VAL ARG ILE PRO ARG PHE PHE met LEU GLN LYS

625 630 635 640 645 650
 TGG TGT AAA GTT CTC AAG GGA AGT GAG GTC ACT TGC CAC CAT TGG AAC AAT TGT CCT ACT CCG CAT TAT TAC TCT AGA GTT ATG ATG GTC
 TRP CYS LYS VAL LEU LYS GLY SER GLU VAL THR CYS HIS HIS TRP ASN ASN CYS PRO THR PRO HIS TYR TYR SER ARG VAL met met VAL

655 660 665 670 675 680
 CTT GAT TCC TAT AGA ATG TCG CTA GAG CTA GTA CAA TTG CCT TTA AGA TAT CCT ATC GGT AGA AGG CAA TTT AAG TCT TTC AAA GCT GAA
 LEU ASP SER TYR ARG met SER LEU GLU LEU VAL GLN LEU PRO LEU ARG TYR PRO ILE GLY ARG ARG GLN PHE LYS SER PHE LYS ALA GLU

685 690 695 700 705 710
 GAA GAC GGC AGT TCC GAT TTG GAA ACT CAA TTG TTA GAT TAT GAT TGC ATC AAA AGA GAT TAT TAC CAT TCT TGC TTA TCG TAC GTT ATA
 GLU ASP GLY SER SER ASP LEU GLU THR GLN LEU LEU ASP TYR ASP CYS ILE LYS ARG ASP TYR TYR HIS SER CYS LEU SER TYR VAL ILE

PvuII 720 725 730 735 740
 TCA GCT GGT GCC GTA AAA TTA GAA GTA ACT ATG GAC GAA ATT CTT GAT ACT TTA GAT GAT GCT GTA ACC AAC AAT GAT ATG AAA GGT ATT
 SER ALA GLY ALA VAL LYS LEU GLU VAL THR met ASP GLU ILE LEU ASP THR LEU ASP ALA VAL THR ASN ASN ASP met LYS GLY ILE

745 750 755 760 765 770
 ATG AAA TCT ATT GAA GAT ATG AAG TCA TTA TTT ATT GAT TCT GGT TCA TTG AAA TCA ACT TGT ACT TGG CTT ACT GCT GAA GTT ATT GAT
 met LYS SER ILE GLU ASP met LYS SER LEU PHE ILE ASP SER GLY SER LEU LYS SER THR CYS THR TRP LEU THR ALA GLU VAL ILE ASP

775 780 785 790 795 800
 CAA ACA AGA CAA GCA TGT GGA CAT GGT TAT TCT TCT TAC AAT GGT TTT GGA AAG GCC TAC AAT GAT TGG GTT GTT CAA TGT ACT TGG GAA
 GLN ALA ARG GLN ALA CYS GLY HIS GLY TYR SER SER TYR ASN GLY PHE GLY LYS ALA TYR ASN ASP TRP VAL VAL GLN CYS THR TRP GLU
 805 810 815 820 825 830
 GGT GAT AAC AAT GTT TTA GGT ATG TCA ATT GGT AAG CCA TTG ATT AAA CAT GTT ATT GAA GTT GTC AAA AGA AAC AAG GTT GTT AAA GGC
 GLY ASP ASN ASN VAL LEU GLY MET SER ILE GLY LYS PRO LEU ILE LYS HIS VAL ILE GLU VAL VAL LYS ARG ASN LYS VAL VAL LYS GLY
 835 840 845 850 855 860
 TCT TCA TCA TTC TTA TCT GAT ACC AAG AAG TAC TTA AAT GAC AAA CCA ATT TTA GAA TCA ATT GAC GAT TTG AAG GAC TTA AGA AAA GTT
 SER SER SER PHE LEU SER ASP THR LYS LYS TYR LEU ASN ASP LYS PRO ILE LEU GLU SER ILE ASP ASP LEU LYS ASP LEU ARG LYS VAL
 865 870 875 HindIII 880 885 890
 TTA GAT GCG ATT GAA GTT TTA ATC ATC AGA TTA CTT ATC AAG CTT TCA TTA ACA ATA GAA GGT AAC GAT GGC GAT TTC GAT CAC GTT GGT
 LEU HIS ALA ILE GLU VAL LEU ILE ILE ARG LEU LEU ILE LYS LEU SER LEU THR ILE GLU ALA ASN ASP GLY ASP PHE ASP HIS VAL GLY
 895 900 905 910 915 920
 GGT GAA ATG GGT ACT CTT TCA AAA TTA AAG GCG CAT CAT TAC TTA TTG AAA GAA TTT TTG GAT AGA ATT GAT ACT TTT GAA AAT AGA GAC
 ALA LEU MET VAL THR LEU SER LYS LEU LYS ALA HIS HIS TYR LEU LEU LYS GLU PHE LEU ASP ARG ILE ASP THR PHE GLU ASN ARG ASP
 925 930 935 940 945 950
 TTC TTT CTT TAT TTG ATT TCT TTA GGT AGA TTA TAT TCT GGT ACA ATC GTC ATT GAA AAA TTT GGT GGC GAA TTT TTA ACT TAT TCA ATC
 LEU VAL MET TYR LEU VAL SER LEU GLY ARG LEU TYR SER ALA THR ILE VAL ILE GLU LYS PHE ALA GLY GLU PHE LEU THR TYR SER ILE
 955 960 965 970 975 980
 TTC CTT ACC AAA TTG GGT GCG AAA TTG ACT TCA AAA TAC ATT CCA GGT TTA TGT AAG GAA ATC AGA CCT AAT GTT GTT CTT TTG ACT GAT
 PHE PRO THR LYS LEU ALA ALA LYS LEU THR SER LYS TYR ILE PRO ALA LEU CYS LYS GLU ILE ARG PRO ASN VAL VAL PRO LEU THR ASP
 985 990 995 1000 1005 1010
 TCC TTC CAA CAA TCC GAT ATG ATC ATT AAC TCA GCA ATT GGT GGC TAC GAT GGT AAT ATC TAC GAA AAT TAC TTC AGA ATT GTC AAG GAA
 SER PHE GLN GLN SER ASP MET ILE ILE ASN SER ALA ILE GLY GLY TYR ASP GLY ASN ILE TYR GLU ASN TYR PHE ARG ILE VAL LYS GLU
 1015 1020 1025 1030 +3587
 AAG AAT CA CCA TTT AAT ACC AAG GGC ACC ATA TTC TGC TGC ATT GGA AGC TAG TGTGACAGACCATCTAAAGATACAAGAGAGAGATTGAGAAAGTTG
 SER ASN PRO PRO PHE ASN THR LYS GLY THR ILE PHE CYS CYS ILE GLY SER ***

growth described, no other genes were contained on the 7.6 kbp Scas-1 fragment unless more than one RNA species of 21s occurs.

The pattern of 21s transcript produced by the Scas-1 gene was studied further using strain 1402 cells grown in minimal medium supplemented with either 1% glucose or 1% maltose. Cells were harvested at 15, 30, 60, and 90 minutes, and at 2, 3, 4, 5, and 24 hours. RNA was extracted, and 20 µg RNA aliquots from each sample were electrophoresed and blotted onto a nitrocellulose membrane. The blots were probed with ³²P-nick translated Scas-1 sequence, washed under stringent conditions and exposed to X-ray film. The result of these hybridizations is shown in Figure 26. In the glucose-grown cells no significant levels of Scas-1 RNA could be detected in any of the samples harvested within the first 5 hours. Only in the 24 hour sample was there a faint band of approximately 21s. In contrast, 21s RNA was detected within the first 15 minutes after shifting to maltose media. The level of transcript increased until a maximum level was reached at 60 minutes, then the amount of transcript decreased to almost non-detectable amounts at 3 to 4 hours, before appearing again at 5 hours and 24 hours. The transcription profile of the Scas-1 gene in cells grown in maltose medium over a 24 hour period can be compared with the in vitro translation profile (see Results Section B(d)) shown in Figure 9. The relative abundance of 120 kDa

Figure 26. Time course Northern analysis of RNA from strain 1402 grown in either glucose or maltose medium and probed with radiolabelled 7.6 kbp Eco RI fragment containing Scas-1 gene. Tracks: a) glucose, 15 min; b) glucose, 30 min; c) glucose, 45 min; d) glucose, 60 min; e) glucose 2 h; f) glucose, 3 h; g) glucose, 4 h; h) glucose, 5 h; i) glucose, 24 h; j) maltose, 15 min; k) maltose, 30 min; l) maltose, 45 min; m) maltose, 60 min; n) maltose, 2 h; o) maltose, 3 h; p) maltose, 4 h; q) maltose, 5 h; r) 24 h. Arrow indicates 21s Scas-1 RNA. Bands indicate location of ribosomal RNA (top to bottom): 25s, 18s. Autoradiograph was exposed for 2 days at -80°C.

a b c d e f g h i j k l m n o p q r

glucoamylase precursor and Scas-1 transcript assayed at the same time points appeared similar, however it is not known exactly why Scas-1 mRNA signal was detected at 24 hours while glucoamylase was not detected by in vitro translation at the same time point.

In the study on the effect of glucose on maltose induced cells (see Figure 27), the addition of glucose to cells actively transcribing Scas-1 RNA resulted in the repression of Scas-1 transcription to non-detectable levels within 30 minutes. Not even degraded Scas-1 RNA was detected in the induced samples given glucose. The turnover of Scas-1 RNA is rapid (with less than 10% remaining after 2 hours growth in the maltose control), nonetheless, its absence after 30 minutes in glucose indicates that glucose very rapidly represses any detectable level of transcription, allowing for the total degradation within the 30 minutes.

The effect of heat-shock on maltose-induced cells was also very striking and is shown in Figure 28. The shift of maltose-induced cells from 30°C to 39°C resulted in the repression of Scas-1 transcription to less than 5% the amount detected in a control sample which was not heat-shocked. It is also noteworthy that heat-shock did not result in the accumulation of Scas-1 RNA larger than the 21s transcript normally seen. This is because processing of messenger RNA precursors is in some cases interrupted by heat-shock, resulting in the observation of larger

Figure 27. Northern analysis of Scas-1 RNA in maltose-induced strain 1402 cells supplemented with glucose. Tracks: a) 0 min (minimal salts washed, pre-shifted control); b) maltose, 30 min; c) maltose, 60 min; d) maltose, 90 min/glucose, 30 min; e) maltose, 120 min/glucose, 60 min; f) maltose, 150 min/glucose, 90 min; g) maltose, 180 min/glucose, 120 min; h) maltose, 90 min; i) maltose, 120 min; j) maltose, 150 min; k) maltose, 180 min. Arrow indicates 21s Scas-1 RNA. Bands indicate location of ribosomal RNA (top to bottom): 25s, 18s. Autoradiograph was exposed for 2 days at -80°C.

a b c d e f g h i j k

Figure 28. Northern analysis of Scas-1 RNA in strain 1402. RNA samples were the same used for the in vitro translation/immunoprecipitations described in Section C. Cells were grown in minimal medium supplemented with 1% of the indicated sugars and /or other conditions described. Tracks: a) no sugar, 30 min; b) no sugar, 60 min; c) no sugar, 90 min; d) maltose, 30 min; e) maltose, 60 min; f) maltose, 90 min; g) maltose heat-shocked from 30-60 min; h) soluble starch, 30 min; i) soluble starch, 60 min; j) soluble starch, 90 min; k) lactose, 30 min; l) lactose, 60 min; m) lactose, 90 min; n) galactose, 30 min; o) galactose, 60 min; p) galactose, 90 min; q) xylose, 30 min; r) xylose, 60 min; s) xylose, 90 min; t) cellobiose, 30 min; u) cellobiose, 60 min; v) cellobiose, 90 min; w) glucose, 30 min; x) glucose, 60 min; y) glucose, 90 min; z) glucose + cAMP, 30 min; a') glucose + cAMP, 60 min; b') glucose + cAMP, 90 min; c') 2-deoxyglucose, 30 min; d') 2-deoxyglucose, 60 min; e') 2-deoxyglucose, 90 min; f') 2-deoxyglucose + cAMP, 30 min; g') 2-deoxyglucose + cAMP, 60 min; h') 2-deoxyglucose + cAMP, 90 min. Arrow indicates 21s Scas-1 RNA. Bands indicates location of ribosomal RNA (top to bottom): 25s, 18s. Autoradiograph was exposed for 2 days at -80°C.

a b c d e f g h i j k l m n o p

q r s t u v w x y z a' b' c' d' e' f' g' h'

transcripts containing introns which are normally spliced out (Lindquist, 1987).

The effect of other sugars on Scas-1 transcription is shown in Figure 28. Again these results do not significantly differ from transcription of glucoamylase RNA measured by in vitro translation. Similar low constitutive levels of 21s transcript, resembling the amounts produced by cells incubated in the absence of carbohydrate, was detected in cells grown in minimal medium supplemented with cellobiose, galactose, lactose, and xylose. RNA isolated from cells grown in minimal medium supplemented with either glucose or 2-deoxyglucose did not contain detectable levels of Scas-1 transcript. As well, the addition of cAMP had no noticeable affect on derepression of Scas-1 transcription.

The combined results leave little doubt that transcription of glucoamylase and transcription of Scas-1 are tightly coupled, and regulated in virtually the same manner.

d) Expression in Bacteria and Other Yeast

Digestion with Sal I releases 1.8 kbp of nonessential DNA from the 5' end of the 7.6 kbp Eco RI fragment, producing a 5.8 kbp Eco RI/Sal I fragment containing the entire Scas-1 gene. This 5.8 kbp fragment was subcloned into the multiple cloning site of the pUC 18 and pUC 19 vectors (Yanisch-Perron et al., 1985) similarly digested with Eco RI and

Sal I. Ampicillin-resistant E. coli strain JM101 transformants were grown and plasmids were extracted. Digestion of these "rescued" plasmids with Eco RI/Sal I followed by agarose gel electrophoresis and U.V. visualization of the ethidium bromide stained gel confirmed that the 5.8 Scas-1 fragment had been subcloned into these vectors. These E. coli transformants were grown in LB broth with ampicillin in the presence and absence of IPTG, an inducer of β -galactosidase transcription, while non-transformed JM101 cells were similarly grown without ampicillin. The cultures were grown for 4 hours at 37°C before total cell-associated and extracellular proteins were analyzed for presence of glucoamylase as described earlier in Schwann experiments (Figures 13-16). In all experiments, no glucoamylase was detected by either immunoblotting or starch plate assay. It therefore does not appear that glucoamylase is produced in E. coli cells carrying the Scas-1 gene.

To test for expression of the Scas-1 gene in heterologous yeasts such as S. cerevisiae or Schiz. pombe, the selectable marker gene, Leu 2 of S. cerevisiae, known to express in both species was used. For these experiments, a 4.15 kbp Pst I fragment containing the LEU 2 gene of S. cerevisiae, from the chimeric cosmid pBTI-1 (Morris et al., 1981) was subcloned into the Pst I site found in the multiple cloning site of pUC 19 possessing the Scas-1 5.8 kbp insert (pUC

5.8), producing pSC 5.8 (Figure 29 A). Attempts to transform this construct into a leu 2- auxotroph of S. cerevisiae failed likely because the plasmid lacked an homologous replication origin. In contrast, about 100 Leu+ prototrophs were obtained from Schiz. pombe strain 2448. These transformants were pooled and grown, along with non-transformed 2448 cells supplemented with 4 μ g/ml leucine using the same conditions to study maltose induction in strain 1402. All cells were glucose adapted prior to shifting to minimal + either 1% maltose or 1% glucose medium. Cells harvested after one hour were used to extract total RNA and total DNA. To determine if Scas-1 DNA was present, the DNA samples were digested with Eco RI and electrophoresed along with corresponding undigested DNA. The DNA was then transferred to a Biodyne membrane and probed with radiolabelled 5.8 kbp Scas-1 fragment under stringent conditions. The result of these hybridizations are shown in Figure 29 B and C. The Scas-1 sequence was not detected in the DNA from non-transformed cells, but was clearly present in the DNA pool from Leu+ transformants, where it appeared to be located in both the high MW DNA fraction, possibly indicating that plasmid integration into the yeast chromosomal DNA had occurred, and in the low MW DNA, signifying the possibility of autonomously replicating plasmid sequence. While electrophoresis did not resolve the main chromosomal DNA from 13.8 kbp supercoiled plasmid, the

Figure 29. Scas-1 expression in yeast Schizosaccharomyces pombe. A. Construction of plasmid pSC 5.8. A 4.15 kbp Pst I fragment harbouring the Saccharomyces cerevisiae LEU 2 gene coding for β -isopropylmalate dehydrogenase was excised from the plasmid pBTI-1 and ligated with pUC 5.8 plasmid recut with Pst I enzyme. Approximately 10% of the RR1 E. coli colonies that were resistant to ampicillin (40 μ g/ml) contained the LEU 2 sequence in addition to the pUC 5.8. This construct called pSC5.8 was used to transform leu 1 (leu 2) auxotrophs of Schiz. pombe strain 2448 to prototrophy. As a rapid screening method, one pool of yeast colonies (about 100) were examined for pSC5.8 content and RNA expression. B. Ethidium bromide stained agarose TAE gel of total genomic DNA. Tracks: a) MW standards; b) 2448 control, uncut; c) 2448 control, Eco RI; d) pooled 2448 transformants, uncut; e) pooled 2448 transformants, Eco RI. C. Southern blot of B probed with radiolabelled 5.8 kbp Eco RI/Sal I fragment containing Scas-1. D. Ethidium bromide stained agarose formaldehyde gel of total RNA. Tracks: e) 2448 control, 60 min. glucose; f) 2448 control, 60 min. maltose; g) 2448 pooled transformants, 60 min. glucose; h) 2448 pooled transformants, 60 min. maltose; i) 1402 60 min. maltose (control). E. Northern blot of D probed with radiolabelled 5.8 kbp Eco RI/Sal I fragment containing Scas-1. Southern and Northern autoradiographs were exposed for 3 days and 5 hours respectively at -80°C .

The diagram illustrates three circular plasmid maps used in molecular cloning:

- PBT I-1 (11.1 kbp):** Contains PBR322, ORI, APR, LEU2, and a 2μ region. Restriction sites include E, H, B, S, P, and XBI. A cloning site is marked with a wavy line and an arrow labeled "CUT".
- PUC5.8 (9.6 kbp):** Contains XBI, B, AV, PV, H, GA, LACZ, and ORI. Restriction sites include XBI, B, AV, PV, H, GA, and LACZ. A cloning site is marked with a wavy line and an arrow labeled "CUT".
- PUC18.19 (13.8 kbp):** Contains LEU2, LACZ, ORI, and various restriction sites. Restriction sites include E, H, B, AV, PV, H, GA, XBI, and LACZ. A cloning site is marked with a wavy line and an arrow labeled "CUT".

B

C

kbp

23

14

abcd e abcd e

Eco RI digested sample hybridized to a species with slower mobility than that of the uncut DNA, which could represent relaxed plasmid DNA or digested chromosomal DNA. In an attempt to rescue the putative autonomous plasmids, total DNA from these Leu 2+ cells was used to transform E. coli strain JM101 to ampicillin resistance. Since a few plasmids were recovered, this indicated that all four genetic functions (E. coli β -lactamase and replication origin, leu 2, and Scas-1) were able to replicate in Schiz. pombe without the presence of a known yeast origin of replication on the original vector. At this time it is not known whether the plasmids are identical to the plasmids used.

To find out whether or not the Scas-1 gene was transcribed in the Schiz. pombe transformants, total RNA from control and transformed strain 2448 samples, along with a positive control of 60 minute maltose-induced total Schwanniomyces RNA were electrophoresed and transferred to nitrocellulose membrane. This Northern blot was then hybridized under stringent conditions to ^{32}P -labelled Scas-1 5.8 kbp sequence. These results are shown in Figure 29 D and E. In the Schiz. pombe control DNA, no Scas-1 sequence was detected. As expected, the 21s Scas-1 transcript was found in the 60 minute RNA of maltose-induced strain 1402 sample. In the glucose grown Schiz. pombe transformed samples, significant transcription was also detected, but not as much as produced in the corresponding maltose-shifted

cells (about 5X). The Scas-1 RNA produced in strain 2448 did not consist of a single 21s RNA as found in strain 1402, but rather a smear containing components of about 21s, 17s and 12.5s in size. The RNA sizes might be accounted for by incomplete splicing of the two putative introns of Scas-1, degradation products of the heterologous message, or a combination of both. These results indicate that the Scas-1 gene is transcribed in Schiz. pombe and that the regulatory sequences of Scas-1 are probably partially recognized as well. Expression of Scas-1 does not appear to be tightly repressed by glucose in Schiz. pombe as in strain 1402. Such differences could also be due to Schiz. pombe repressor-Scas-1 gene copy ratio, resulting from autonomously replicating plasmid. These same total RNA samples were translated in vitro and immunoprecipitated using the anti-glucoamylase sera. Compared to the non-transformed controls, no significant difference was observed in the transformed samples grown in either glucose or maltose, and no differentially expressed proteins were immunoprecipitated. This means that either the Scas-1 gene mRNA does not code for the 120 kDa preglucoamylase, or that this RNA is not translatable because it is not intact. Precise mapping of the transcripts along the Scas-1 gene would help clarify to what degree transcription and processing fidelity has taken place in Schiz. pombe.

These results are in agreement with earlier attempts to clone the Schwann. castellii glucoamylase directly into S. cerevisiae and Schiz. pombe by screening for expression of enzyme activity (Dowhanick and Seligy, unpublished results). Cloning and expression of Schwann. castellii glucoamylase gene(s) was attempted by construction of cosmid libraries containing strain 1402 DNA approximately 30 kbp in size (randomly cut by Sau 3A) ligated into the yeast shuttle cosmid vector pBTI-1 (Morris et al., 1981). Putative leu 2 auxotrophic yeast transformants selected for ability to grow in the absence of leucine were screened by starch plate assay. No clones expressing glucoamylase were recovered out of the 10 trial attempts involving approximately 2.5×10^4 transformants. These results suggested that the glucoamylase gene may not directly express in heterologous yeasts because of differences in genetic recognition signals.

e) Homologies to Known Genes

To test for the presence of sequence homologous to the Scas-1 gene in other yeast and fungi known to possess amylolytic genes, a Southern blot was prepared containing Eco RI digested total genomic DNA from the following: Aspergillus nidulans, A. niger, A. oryzae, Candida pelliculosa, C. wickerhamii, Pichia stipitis, Rhizopus arrhizus, R. formosensis, R. oligosporus, Saccharomyces

cerevisiae, S. diastaticus, Saccharomycopsis fibuligera,
Schwannomyces castellii (control), and Thermomyces
longinosus. The blot was prehybridized, hybridized to ^{32}P -
 nick translated Scas-1, washed under non-stringent
 conditions and exposed to X-ray film. The results are shown
 in Figure 30. Under non-stringent conditions, Scas-1
 sequence hybridized weakly to DNA from all of the species
 tested. Binding of probe was not as well defined nor as
 extensive as with the DNA of Schwann. castellii strain 1402
 used as a control. However binding in all cases occurred to
 DNA fragments approximately 6-9 kbp in size. Scas-1
 sequence appeared to bind to two bands in Saccharomycopsis
fibuligera and Aspergillus oryzae. Binding was stronger in
Saccharomyces diastaticus (known to possess several, up to
 four different glucoamylases) than in S. cerevisiae (known
 only to possess a sporulation-specific glucoamylase),
 although both were extremely weak. Binding appeared
 strongest to Saccharomycopsis fibuligera and Aspergillus
oryzae compared to the amount of DNA loaded. Rewashing the
 filter under stringent conditions resulted in removal of all
 heterologous binding except for very weak binding to
Saccharomycopsis fibuligera and Aspergillus oryzae. These
 results indicate that there is very little homology between
 the Scas-1 sequence and the various yeast and fungi tested.
Scas-1 sequence was shown to bind strongly to 10 kbp
Hind III digested Schwann. alluvius DNA and this sequence is

Figure 30. Southern analysis of Eco RI digested genomic DNA isolated from various yeast and fungi and probed with radiolabelled Scas-1 under non-stringent conditions. A. Ethidium bromide stained agarose-TAE gel electrophoresed with the digested DNA samples. B. Autoradiograph of Southern blot of A after hybridization to probe. Tracks: a) Schwann. castellii strain 1402 (control); b) Saccharomycopsis fibuligera strain 1572; c) S. fibuligera strain 1573 ; d) Schwann. alluvius, e) S. diastaticus strain 2472, f) S. distaticus strain 2473, g) S. cerevisiae, h) Rhizopus arrhizus, i) R. formosae, j) R. oligosporus, k) Candida pelliculosa, l) C. wickerhamii, m) Pichia stipitis, n) Thermomyces longinus, o) Aspergillus nidulans, p) A. niger, q) A. oryzae. Arrows indicate regions of Scas-1 binding. Bands indicate location of MW standards (top to bottom): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0. Autoradiograph was exposed for 20 hours at -80°C

presently being cloned into the λ Ch30 vector (Prakash, Dowhanick, and Seligy, unpublished).

Attempts have also been made to identify glucoamylase-related sequences in strain 1402 genomic DNA by hybridization under nonstringent conditions with available heterologous probes. The only available glucoamylase probe was the sporulation-specific glucoamylase from S. diastaticus (Erratt and Nasim, 1986). When this probe was hybridized under stringent conditions to Bam HI digested genomic DNA from S. diastaticus (NRCC strain 2472) the 3.9 kbp sporulation-specific glucoamylase was detected, as well as a 4.5 kbp sequence. The latter fragment was cloned using λ Ch30 as vector, and isolated using the S. diastaticus sporulation-specific glucoamylase as a probe (Dowhanick and Seligy - unpublished). Restriction analysis of this cloned sequence followed the same pattern as the STA I (DEX 2) gene reported by Yamashita and Fukui (1984). Both of the glucoamylase genes from S. diastaticus were radiolabelled and hybridized to Southern blots of Bam HI, Eco RI, and Hind III digested strain 1402 genomic DNA (under non-stringent conditions) and to similarly digested samples of strain 2472 genomic DNA (under stringent conditions). The results of these hybridizations are shown in Figure 31. While the 3.9 and 4.5 kbp glucoamylase sequences revealed a family of related sequences in the S. diastaticus genome, no significant hybridization to strain 1402 DNA was detected.

Figure 31. Southern analysis of restriction endonuclease digested genomic DNA from Schwann. castellii strain 1402 and S. diastaticus strain 2472. The ethidium bromide stained gel (top) was blotted and probed with radiolabelled 3.9 kbp sporulation-specific glucoamylase (A) or 4.5 kbp DEX 2/STA 1 sequence of S. diastaticus (B) under non-stringent conditions (bottom). Tracks: a) and g) 1402 Bam HI; b) and h) 1402 Eco RI; c) and i) 1402 Hind III; d) and j) 2472 Bam HI; e) and k) 2472 Eco RI; f) and l) 2472 Hind III. Bands indicate MW standards: (top to bottom) 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6. Autoradiograph was exposed for 2 days at -80°C.

Therefore, it appears that strong homology does not exist between the glucoamylases of S. diastaticus and Schwann. castelli.

Two other glucanase probes were available for hybridization to strain 1402 DNA. These included the 3.4 kbp Eco RI fragment containing the entire α -amylase Amy II gene from A. oryzae (Gines, 1987; Gines and Seligy - submitted), and the 0.52 kbp Eco RI cDNA fragment containing the conserved sequence for the β -glucosidase of Schizophyllum commune (Moranelli et al., 1986). Hybridization of these radiolabelled probes to genomic blots of strain 1402 DNA under non-stringent conditions failed to probe the 7.6 kbp Eco RI Scas-1 gene. These results rule out the possibility that the Scas-1 gene codes for either an α -amylase or a β -glucosidase.

IV. DISCUSSION

A) General Properties of Schwanniomyces castellii Growth on Amylosic Substrates

In the past, several studies on amylolytic expression in Schwanniomyces yeasts have been carried out (see Introduction). These studies did not yield any molecular detail on either the biosynthesis or the modes of induction/repression of any of the enzymes involved. This thesis addresses the induction/repression problem by introducing a nutrient-shift strategy to synchronize the cell population response prior to molecular analysis. Glucoamylase expression of Schwann. castellii strain 1402 was examined over a 24 hour period by shifting cells from glucose supplemented minimal medium to either maltose or soluble starch medium. Under the conditions of growth specified, shifted-exponentially growing cells completed a cell doubling, while shifted stationary phase cells did not. The inability of stationary cells to enter exponential phase is most likely due to the limiting amount of nutrient given to them. However, all cultures were metabolically active as seen by the utilization of their respective carbohydrate sources.

From the analysis of glucoamylase activity measured by the liberation of free glucose, glucoamylase was expressed to a greater extent and significantly faster in maltose than

in soluble starch medium. Exponentially growing cells shifted to maltose medium expressed glucoamylase activity at all stages of growth with maximum activity appearing at mid-log phase. Similarly prepared cells shifted to soluble starch medium exhibited glucoamylase activity after mid-log phase, with maximum activity occurring in either late-log or early stationary phase. Additional time points between 4 and 5 hours would be needed to determine the exact stage at which maximal activity occurred. Carbon catabolite repression of glucoamylase occurred with cells grown in glucose media, since only a very low, basal level of liberated glucose (less than 5% of that of cells grown in maltose medium) could be detected by enzymatic assay. This very low level of liberated glucose could have been due to the action of low level constitutively synthesized α -amylase produced by strain 1402 (Sills *et al.*, 1984b) as it has been demonstrated that α -amylase isolated from this yeast has a marginal capability of hydrolyzing maltose (Sills *et al.*, 1983).

B) Glucoamylase Expression in Schwanniomyces castellii

By the use of polyclonal anti glucoamylase antibody made to HPLC purified 146 kDa active glucoamylase, it was demonstrated that two polypeptides are actually detected in both cellular and extracellular preparations of Schwann. castellii in response to maltose. One is the same size as

the mature, 146 kDa glucoamylase found in both starch and maltose cultures. The second is a 78 kDa polypeptide which has no amylolytic activity, and is believed to be a breakdown product of the glucoamylase. In the time-course glucoamylase expression studies presented in Fig. 5, the 78 kDa product appeared on or just prior to the 146 kDa enzyme. Competing the anti-glucoamylase sera with total proteins from glucose-repressed cells effectively eliminated detection of this major polypeptide, as well as other minor components of low molecular weight, while not affecting detection of the 146 kDa glucoamylase. A similar-sized breakdown product (78 kDa) detected in the column-purified control glucoamylase samples (Figures 4 and 5) was similarly effected by precompetition of the anti-serum (Figure 6). This observation is not unusual considering that the antibody used in these experiments is polyclonal. Immunization by injection of a purified antigen such as the purified 146 kDa glucoamylase into an animal would likely stimulate the synthesis of an heterogeneous population of antibody, each antibody clone differing from the other in both affinity and specificity of epitope recognition and interaction (Hurn and Chantler, 1980). This would explain why the 78 kDa protein could be competed out relative to the 146 kDa protein. The pattern of expression of the 146 kDa glucoamylase related products revealed in the cell concentration studies (Fig.7) further indicates that at low

cell concentrations only the 138 kDa and 146 kDa products are present; at high cell concentrations, several major (120, 105, 88, and 66 kDa) as well as minor (115, 105, 88, 78, 59, and 53 kDa) products are produced whose patterns differ in the cell lysates and culture supernatants. These are likely early processed or turn over, degradation products of glucoamylase. Consistent with this interpretation, it was observed that purified 146 kDa glucoamylase often undergoes autodegradation, yielding polypeptides mainly 66, 78, and 88 kDa products. Other minor ones include 120 and 105 kDa.

Glucoamylase expression was studied in Schwann, castelli strain 1402 by growing cells in the presence or absence of a variety of substrates and under different conditions in order to develop a more comprehensive understanding of the mechanism of its regulation. This expression was monitored at both the level of transcription and translation by immunodetection of in vitro and in vivo translated products, respectively. The information gained from these experiments has helped to hypothesize the genesis of the 146 kDa mature glucoamylase as well as possible mechanisms of its regulation. There is no doubt that expression of glucoamylase is highly regulated in response to its source of carbohydrate, and that this regulation occurs mainly at the level of transcription. In this respect, glucoamylase expression is similar to that of invertase of S. cerevisiae.

(Carlson and Botstein, 1982), glucoamylase of both A. niger (Nunberg et al. 1984) and A. awamori (Innis et al. 1985), and α -amylase of A. oryzae (Erratt et al. 1984).

Studies were conducted on the glucoamylase precursor by in vitro translation of isolated glucoamylase messenger RNA as was done previously for the α -amylase of Aspergillus oryzae (Erratt et al. 1984) and the cellulases of Schizophyllum commune (Willick and Seligy, 1985). My results from immunoprecipitation of the glucoamylase molecule translated in vitro from a rabbit reticulocyte cell-free system revealed that the nascent glucoamylase precursor is 110-120 kDa in size. Immunoanalysis of cell-associated and extracellular products detected in vivo further indicated that this nascent 120 kDa polypeptide must be rapidly converted to two stable forms. The first modification results in the synthesis of the cell-associated 138 kDa precursor; the second modification results in formation of the 146 kDa mature protein, most of which is secreted into the medium. The additional 26 kDa is most likely the result of post-translational modification such as glycosylation. However, probably a secretion signal peptide found at the N-terminus of the 120 kDa nascent peptide is also removed. In a review of fungal glucoamylases (Manjunath et al., 1983), glycosylation was shown to account for anywhere from 3-30 % of the total MW of this enzyme. If the size difference noted for the strain 1402 glucoamylase

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studied here is due to glycosylation (17.8%), then the data are supported. These post-translational modifications likely occur at specific stages along the secretory pathway as has been described for S. cerevisiae (see reviews by Scheckman, 1982; Novick and Scheckman, 1982). A question that remains unanswered at this time is what are the exact post-translational modifications that convert the nascent, 120 kDa to the mature, 146 kDa protein. What I have shown is that there is at least one intermediate step (138 kDa). Additional information may be obtained by studying the biosynthesis of glucoamylase in the presence of an inhibitor of glycosylation such as tunicamycin (Willick et al., 1984; Willick and Seligy, 1985).

C) Correlated Expression of Glucoamylase and Scas-1 Gene

Information obtained on the induction of glucoamylase synthesis and expression in Schwann. castellii strain 1402 was used to construct a cDNA library, which when enriched by competing out common glucose RNA sequence before probing M13 cDNA plaques by hybridization, produced two clones containing the same sequence for an mRNA induced by maltose and repressed by glucose. The induced mRNA is approximately 21s or $3.15-3.25 \times 10^3$ nucleotides in size, and could code for a nascent protein of about 110-120 kDa. The DNA sequence responsible for this message was located on a 7.6 kbp Eco RI fragment which was subsequently cloned using the λ bacteriophage vector Charon 4A. Although the DNA sequence

and transcription region has been determined, because of lack of available amino acid sequence for the 146 kDa glucoamylase no definitive identification was made at this time (see Sections D and E for discussion of Scas-1 gene organization and homology). Nevertheless, all of the Northern analyses clearly established that the pattern of Scas-1 transcription is similar to that of the nascent 120 kDa (immunoprecipitable) glucoamylase translated in vitro using the same RNA samples.

In the maltose induction experiments, the respective products were observed within 15 minutes after transfer from glucose to maltose-supplemented media, reaching a maximum at 60 minutes, decreasing to very low levels at 3 hours and 4 hours, and increasing again by 5 hours. While Scas-1 RNA was detected at 24 hours, the in vitro translation products were in general not clearly detectable. While the exact reason for this is not known, this observation may be the result of partial degradation of the mRNA to an untranslatable state. Under similar growth conditions, in vivo translated glucoamylase appeared to undergo partial degradation by 24 hours. Therefore, the decrease in levels of Scas-1 and glucoamylase message after 2 hours could represent the degradation of transcript due to carbon catabolite repression. Loss of repression (which had taken place by 5 hours) could have been due to reduction of intracellular glucose levels to threshold induction levels.

It has been reported in Saccharomycopsis (Endomycopsis) fibuligera that a build up of cyclic AMP during glucose repression of glucoamylase expression was responsible for derepression of the enzyme (Afanas'eva et al., 1978). However, using the same concentrations of cAMP as described by this group, derepression by cAMP was not observed in strain 1402 cells grown in minimal medium supplemented with either 1% glucose or 1% 2-deoxyglucose. Studies were also conducted on strain 1402 grown in non-supplemented minimal medium (i.e. without carbohydrate) as well as minimal media supplemented with either 1% (w/v) cellobiose, lactose, 2-deoxyglucose, galactose, xylose, and soluble starch. In all cases where glucoamylase was immunodetected, Scas-1 RNA was also present. Most interesting was the detection of both glucoamylase and Scas-1 RNA in medium lacking carbohydrate. This implies that at the molecular level, both glucoamylase (RNA) synthesis and Scas-1 transcription are inducible, constitutive-like and glucose-repressible.

The effect of heat shock on the expression of glucoamylase and Scas-1 RNA was also investigated using cells induced by maltose. By immunodetection of cell-associated and extracellular glucoamylase, a thirty minute heat shock at 39°C arrested synthesis of the 138 kDa precursor protein, which in turn resulted in a depletion of extracellular 140 kDa protein. Analysis by in vitro translation and immunoprecipitation revealed that the heat

shock had resulted in either termination of transcription or accelerated degradation of the 120 kDa glucoamylase message, at the same time inducing transcription of other mRNAs. Judging by the sizes of the heat shock induced proteins, they appear to be comparable to those documented in the yeast *S. cerevisiae* (McAlister *et al.*, 1979; McAlister and Finkelstein, 1980). Northern analysis of the same heat shocked total RNA again revealed that Scas-1 message had been similarly affected. These results demonstrate that repression of glucoamylase and Scas-1 mRNA can be brought about by heat shock just about as rapidly and as effectively as by the addition of carbon catabolite repressors such as glucose and 2-deoxyglucose.

D) Properties of Scas-1 Gene

The putative size of the Scas-1 gene is consistent with that expected for the 110-120 kDa glucoamylase precursor. Since amino acid sequence of glucoamylase is not available, it remains to be determined whether or not the Scas-1 gene codes for this enzyme. Transfer of the Scas-1 gene into *Schiz. pombe*, albeit successful, failed to produce intact mRNA which could be studied by *in vitro* translation and immunoprecipitation to verify that the gene codes for glucoamylase. Eventually when glucoamylase amino acid sequence analysis becomes available, the identity of Scas-1 will be known. If Scas-1 does not code for glucoamylase,

the amino acid sequence of the latter could be used to produce a synthetic oligonucleotide probe to clone the corresponding glucoamylase gene and study its relationship to Scas-1. The tight correlation of expression of Scas-1 and glucoamylase transcripts suggests that these RNAs are one and the same and that Scas-1 is not a trans acting factor which acts on the glucoamylase to promote its expression. Such a protein would be expected to be made at a slightly earlier point in time.

Since Northern analysis of Scas-1 in Schiz. pombe indicates that most of the RNA is not intact, it seems that a full-length cDNA clone of this gene is needed to further study heterologous expression. As well, because of the stringent regulation of expression of Scas-1 in the presence of glucose and maltose, the knowledge and use of both the yet unknown putative "trans" acting proteins (activator and repressor) and "cis" acting sequences of Scas-1 would be beneficial for production of heterologous proteins in this already useful industrial organism. Furthermore, a mutant of strain 1402 which is derepressed for amylolytic activity has been isolated (Sills et al., 1984). Studies of Scas-1 transcription in comparison to glucoamylase expression in this strain may be useful to determine whether or not regulation of these products had been affected.

E) Comparison to Glucoamylase of Saccharomyces diastaticus

Although Table 2 lists the molecular weights of glucoamylases from various yeasts and fungi, little is known about their expression at the molecular level. More recent comparison of the DNA sequence of glucoamylases from A. awamori (Nunberg et al., 1984), and A. niger (Svensson et al., 1983), with S. diastaticus (Yamashita et al., 1985) have revealed that they are a diverse family of proteins, bearing little intergenic homology (about 30% - Mackay et al., 1985). Comparison of amino acid sequences of glucoamylases from Aspergillus, Rhizopus, and Saccharomyces gave an overall homology of only 25-36% (Tanaka et al., 1986), and consensus regions from these three species were not similarly found in Scas-1 (data not shown). As well, in this study it was shown that the STA 1 glucoamylase from S. diastaticus was not homologous enough to hybridize to Schwann. castelli strain 1402 glucoamylase sequence, even under non-stringent conditions. Since the coding region of these genes is much smaller (about 60%) than that predicted for the glucoamylase nascent peptide (110-120 kDa), it would not be surprising that the primary structure of the glucoamylase of Schwanniomyces and its regulation of expression would differ from other glucoamylases reported.

The best characterized glucoamylases in yeast (and possessing two nomenclatures) belongs to the DEX (Erratt and Stewart, 1981)/STA (Tamaki, 1978) family of S. diastaticus.

They comprise three genes: DEX 1 (allelic to STA 2), DEX 2 (allelic to STA 1), and DEX 3 (allelic to STA 3) which express during vegetative growth, and one (SGA) which expresses during sporulation (Collona and Magee, 1978; Erratt and Nasim, 1986) and appears to be related to the sporulation-specific glucoamylase of S. cerevisiae (Yamashita and Fukui, 1985). Transcriptional control of the DEX 1(STA 2) was recently investigated using the respective cloned gene as a probe (Pretorius et al., 1986). The growth conditions and medium formulation differed considerably from those used in the present study, making direct comparisons difficult. In S. diastaticus, as well as strain 1402, glucoamylase expression was regulated at the level of transcription, and was dependant on the growth medium and carbon source. In richer medium such as YP (1% yeast extract, 2% bacto peptone) glucoamylase synthesis was greater (up to 4X) than in synthetic complete (SC - Sherman et al., 1972) medium. Maximum activity came from YP + 3% glycerol + 2% ethanol (100%). In contrast to strain 1402, in S. diastaticus galactose was as good an inducer of glucoamylase (61%) as maltose (60.2%) in YP medium, and glucose produced significant levels (30%). Also in contrast to strain 1402, starch was a better inducer (89%) than maltose. In a similar experiment to those involving strain 1402, the addition of glucose to glycerol/ethanol induced cells resulted in a reduction of glucoamylase expression.

Presence of the STA 10 gene product in Saccharomyces spp. resulted in repression of glucoamylase expression at the level of transcription, and the authors (Pretorius et al., 1986) felt that the STA 10 product was either a negative regulatory protein or an inactive form of a positive regulatory protein. While no such protein has been identified in Schwanniomyces spp., the data indicating that glucoamylase is regulated is consistent with the existence of a similar gene product.

In contrast to results obtained with strain 1402, synthesis and secretion of S. diastaticus glucoamylase occurred in YP medium supplemented with 2% glucose (Pretorius et al., 1986). However, these authors noted that while glucoamylase synthesis is severely glucose-repressed in Neurospora crassa (Sigmund et al., 1985), significant amounts were produced by A. awamori in glucose-containing media. This indicates that carbon catabolite repression of glucoamylase varies significantly among different yeast and fungi, just as the gene sequences differ. It therefore appears unlikely that accurate comparisons of glucoamylase induction and repression can be made among different species and genera unless growth and medium conditions are made identical and all genes and probes for the respective glucoamylases are available.

F) Model for Regulation of Glucoamylase Expression in Schwanniomyces castellii

Data presented in the Results revealed three levels of glucoamylase expression in Schwann. castellii: 1) an induced expression; 2) a constitutive-like expression; and 3) a repressed expression. Induced glucoamylase expression was observed when glucose-adapted cells were shifted to maltose. This induction occurred very rapidly (within 30 minutes), however it could be completely repressed as quickly as it was induced by either heat shock or addition of glucose or 2-deoxyglucose to the medium. A much lower, but constitutive level of glucoamylase expression (approximately 5% of the induced level) was observed when glucose-adapted cells were shifted to minimal supplemented medium possessing sugars other than maltose, glucose or 2-deoxyglucose, or in the absence of exogenous carbohydrate. This constitutive expression also occurred very quickly, within 30 minutes after transfer to the respective medium. Nearly identical results were obtained for the Scas-1 gene.

Since both glucoamylase and Scas-1 regulation were shown to occur at the level of transcription in Schwann. castellii, a model based on current understanding of gene regulation in S. cerevisiae can be proposed. The model which is shown in Figure 32 supposes that the glucoamylase (or Scas-1) gene possesses a positive activation site (PAS)

Figure 32. Schematic model illustrating glucoamylase regulated expression in the yeast Schwanniomyces castellii. In the presence of glucose (> 3 mM), glucoamylase (GA) gene repressor protein (R) is activated by either de novo synthesis or post-translational modification. This trans-acting repressor binds to "cis", negative activation sequences (N) located between the promoter (P) and translational start signal (X), blocking all transcription of the 21s, GA mRNA (.....). Alternatively, R may interact with the trans-activating product (A) which normally enhances transcription by binding at the "cis", upstream activation sequence (U). In the absence of glucose (< 3 mM) and presence of other types of sugars and/or polymerized amylose, a low level of ~~21s~~ GA mRNA is made (____) which is processed and translated into 120 kDa preglucoamylase. Pre-GA is processed intracellularly to a 138 kDa polypeptide intermediate and then to the 146 kDa, mature, extracellular, active GA. The 146 kDa enzyme acts on starch and/or maltose to release glucose. Maltose, which is normally produced from starch by the action of other amylolytic enzymes (see Table 1) is taken up by the yeast cell and signals the production of A product, thus enhancing the level GA mRNA and GA production. High levels of GA result in rapid conversion of starch or maltose to glucose which then induces the shut-off of GA transcription.

- - α - AMYLASE
- - β - AMYLASE
- - GLUCOAMYLASE
- - ISOAMYLASE/PULLULANASE

upstream of the promoter, and a negative activation site (NAS) downstream of the promoter either before or after the site of initiation of transcription. In the absence of either inducer or repressor promoting sugar, the transcription promoter is capable of low-level, constitutive-like synthesis, due to the absence of interactions at either PAS or NAS. As well, two genes coding for trans-acting regulatory proteins are hypothesized: one gene codes for an activator protein (A) and the other gene codes for a repressor protein (R). In the presence of maltose, this disaccharide enters the cell via a maltose permease transport receptor molecule located in the cell membrane. The maltose molecule first triggers either modification or de novo synthesis of the activator gene. The activator protein is now available to bind the "cis" sequences at PAS, resulting in enhanced glucoamylase transcription. Glucoamylase transcript is then translated into the 120 kDa nascent precursor, modified to the 138 kDa intermediate precursor, and further modified to the 146 kDa mature, active form which is secreted into the medium and can now degrade maltose or starchy derivatives to glucose.

Entry of glucose or 2-deoxyglucose into the cell via a glucose transport system at concentrations exceeding 3 mM induces the modification or de novo synthesis of repressor protein which in turn binds to the NAS, resulting in complete repression of glucoamylase transcription. The PAS

and NAS flank the promoter region. When the concentration of glucose or 2-deoxyglucose falls below 3 mM, the repressor protein is diluted out either by cessation of synthesis or proteolytic degradation so that binding to the NAS no longer occurs. It is not certain at this time whether or not 2-deoxyglucose is metabolized, but it is known that this glucose analog is transported and phosphorylated in *S. cerevisiae*, accumulating in intracellular pools as a mixture of free sugar and several derivatives (Franzusoff and Cirillo 1982). If 2-deoxyglucose is not metabolized, derepression might occur only if the cells are fed a metabolizable sugar, allowing for growth and eventual dilution to non-significant levels.

The in vivo protein labelling data shown in Figure 2 reveals that in glucose exposed cells, there are two abundant proteins (43 kDa and 47 kDa) that show up early in the experiment. In maltose shifted cells, two proteins measuring 56 kDa and 66 kDa are synthesized before significant levels of glucoamylase are detected. While the identity of these induced proteins are not known, the appearance of the 56 or 66 kDa protein, serving as putative activator proteins, or the 43 and 47 kDa proteins serving as repressor proteins is consistent with the suggested model for regulation of glucoamylase expression.

Cells grown (or incubated) in the absence of glucose and maltose would not induce synthesis of either repressor or

activator proteins. Under these conditions, neither induction nor repression would occur, leaving the glucoamylase promoter to facilitate a constitutive-like expression of glucoamylase transcript. Consistent with the data, the model predicts that any build up of glucose concentration due to the hydrolysis of maltose or starch by glucoamylase should result in fluctuations in glucoamylase transcription as observed in Figure 12. These results are also consistent with proposal of Sills *et al.* (1984b) to explain the induction of α -amylase in the presence of starch (and discussed in Section A of Results). In their studies, low level constitutive synthesis of α -amylase in the presence of starch would result in the breakdown of this macromolecule to oligosaccharides as well as to maltose. The effectiveness of other starchy oligosaccharides to serve as inducers of glucoamylase synthesis has not been determined. It must be stated that while the proposed model of glucoamylase regulation fits the data obtained in these studies, at the level of transcription determined by *in vitro* translations (Figures 17-19), there were no clear candidates to serve as putative trans-acting activator or repressor proteins. Comparison of the nascent synthesized proteins of maltose and glucose treated cells showed only one obvious, relatively abundant protein, the 110-120 kDa pre-glucoamylase. Future experiments could focus on determining what proteins serve as regulatory agents in

amylolytic enzyme production in this yeast.

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