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CONSTRUCTION OF A GENE EXPRESSION MODEL IN MUCOR
(TRANSFORMATION OF M. racemosus WITH A
TEF1/hGH FUSION PLASMID)

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CONSTRUCTION OF A GENE EXPRESSION MODEL IN *MUCOR*

(TRANSFORMATION OF *M. racemosus* WITH A TEF1/hGH FUSION PLASMID)

By

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ABSTRACT

CONSTRUCTION OF A GENE EXPRESSION MODEL *IN MUCOR*
(TRANSFORM M. racemosus WITH A TEF1/hGH FUSION PLASMID)

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The promoter is one very important element of a high level expression vector. To identify a suitable promoter for high level expression of heterologous genes, the function of the Mucor racemosus TEF1 promoter was investigated in this model fungal protein expression system. A model plasmid was constructed by fusing the human growth hormone (hGH) gene with the TEF1 promoter. The hGH gene was inserted into pMR1-3 at a position adjacent to the TEF1 promoter. The correct structure of plasmid pMYY1 was confirmed and used to transform M. racemosus R7B. Southern analysis showed that pMYY1 was present in the cells with no DNA rearrangements. However, Northern analysis did not detect an hGH gene fusion transcript in Mucor cells. These results suggested that the TEF1 promoter was not capable of driving the high level expression of a heterologous gene. Future research will investigate additional factors required for the function of TEF1 promoter.

Dedicated to my dear parents,

R.C. Yen and F.H. Yen.

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TABLE OF CONTENTS

		PAGE
LIST OF TABLES		vi
LIST OF FIGURES		vii
I.	INTRODUCTION	1
II.	HISTORICAL REVIEW	4
	Genetic Modification of Enzymes Used in the Food Industry	4
	Enzyme Uses in Food Areas	4
	Conventional Sources of Enzymes	6
	Enzymes production via Recombinant DNA Technology	8
	Heterologous Gene Expression Systems	13
	E. coli as a host	14
	Bacillus subtilis as a host	16
	Yeast as a host	17
	Filamentous fungi as a host	20
	Transformation System	21
	Transformation of Bacterial Cells	23
	Transformation of *S. cerevisiae*	23
	Transformation of Filamentous Fungi	24
	Mucor Heterologous Protein Expression/Secretion System	26
III.	MATERIALS AND METHODS	29
	Microbial Strains	29
	Plasmids	29
	Chemicals	30
	Growth Medium and Growth Conditions for *E. coli*	37
	Transformation of *E. coli*	37
	Colony Hybridization for the Identification of Recombinant Plasmids in *E. coli*	39
	Isolation of Plasmid DNA from *E. coli*	40
	Growth Conditions for *Mucor*	41
	Transformation of *Mucor*	42
	Single Colony Isolation of *Mucor* Transformants.	44
	Isolation of Genomic and Plasmid DNA from *Mucor*	45
	Restriction Endonuclease Analysis and Gel Electrophoresis of DNA	47
	Preparation of RNA from *Mucor*	47
	Gel Electrophoresis of RNA	49
	Southern and Northern Analyses of Nucleic Acid.	49
	Nuclease-S1 Mapping of RNA	51
	β-Galactosidase Assay	52

IV. RESULTS 54
(1) TEF1/lacZ Fusion Plasmid 54
Construction of Gene Fusion Plasmids 54
Southern Analysis of DNA Isolated from *Mucor* Transformants 57
Northern Analysis of RNA Extracted from *Mucor* Transformants- pMF25 and pMF29 67
S1-Nuclease Mapping of RNA Extracted from Transformants 70
β-Galactosidase Assay 73
(2) TEF1/hGH Fusion Plasmid 74
Construction of Gene Fusion Plasmids 74
Isolation and Analysis of Constructed Plasmids 77
Transformation of *Mucor racemosus* R7B with pMYY1 and pMR1-3 87
Southern Analysis of DNA Extracted from *Mucor* Transformants 88
Northern Analysis of RNA Extracted from *Mucor* Transformants 89

V. DISCUSSION 97
VI. SUMMARY 104
VII. REFERENCE 105

LIST OF TABLES

Table		page
1.	Major enzymes used by the food industry	5
2.	Food processing enzymes and food additives used in the U.S. food industry that benefit from genetic engineering technology	11

LIST OF FIGURES

figure page

1. Restriction map of plasmid pLeu4 31
2. Restriction map of plasmid pMR1-3 33
3. Restriction map of plasmid pOGH 35
4. Construction of plasmid pMF14 55
5. Construction of plasmid pMF25 and pMF29 58
6. Restriction map of plasmid pMF25 60
7. Restriction map of plasmid pMF29 62
8. Southern analysis of genomic DNA isolated from M. racemosus R7B, pLeu4 transformants, pMF25 and pMF29 transformants using a ^{32}P-labeled 6.2 Kb SalI fragment (lacZ) and pUC8 fragment as probes 65
9. Northern analysis of total RNA purified from M. racemosus R7B, a pLeu4 transformant, a pMF25 transformant and a pMF29 transformant using a ^{32}P-labeled 6.2 Kb SalI fragment containing the lacZ gene as the probe 68
10. S1-nuclease analysis of total RNA purified from M. racemosus R7B, a pLeu4 transformant, a pMF25 transformant, and a pMF29 transformant 71
11. Construction of plasmid pMYY1 75
12. Construction of plasmid pMYY1 (nucleotide sequence). 78
13. Colony hybridization with an hGH gene fragment as probe 81
14. Restriction analysis of plasmid pMYY1 83
15. Restriction map of plasmid pMYY1 85
16. Southern analysis of genomic DNA isolated from four pMYY1 transformants and pMR1-3 transformant (control) using a ^{32}P labeled 1.8 Kb AatII fragment and 1.9 Kb HindIII fragment as probes 90

17. Northern analysis of total RNA isolated from four pMYY1 transformants and a pMR1-3 transformant using a ^{32}P labeled 1.8 Kb AatII fragment containing the the hGH gene and a 1.9 Kb HindIII fragment containing the Leu1 gene as probes 94

INTRODUCTION

Proteins play a very important role in industry. They have gained considerable attention in many industrial areas because enzyme-catalyzed reactions are clearly superior to purely chemical reactions in most cases. They are faster, more specific and safer. It was estimated that worldwide enzyme sales will reach $ 1.5 billion by 1990 (Knorr and Sinskey, 1985). Approximately 48% of all sales correspond to protease. Although the need for enzymes is continuously increasing, the quantity of enzymes produced by industrial microorganisms is too low to satisfy this demand. In order to increase the yields of enzymes and decrease the expense of producing industrial enzymes, areas of intensive research are microbial strain improvement, enzyme/cell immobilization and enzyme stability enhancement.

Recombinant DNA technology is a significant new approach to strain improvement because it provides the tools to bring together genes from different organisms into one organism for increasing product yields or producing entirely new substances. Significant progress has been achieved using recombinant DNA technology for the production of nonmicrobial products in microbes. For example, insulin, somatostatin, human growth hormone, virus vaccines, and interferon have all been produced using recombinant DNA

technology (Bollon, 1983). There are several different microbial systems for heterologous protein expression under study such as E.coli , Bacillus subtilis and Saccharomyces cerevisiae (Lin, 1986). Recently, considerable research effort has focused on the molecular genetics of filamentous fungi. The filamentous fungi are extremely attractive as potential hosts for expression of cloned genes for use in industry, since they naturally secrete vast quantities of protein and have the ability to successfully express a variety of heterologous genes without the need for extensive DNA manipulation (Dickinson, et al., 1987 and Van Brunt, 1986).

Mucor, a member of the filamentous fungi, is an excellent target for genetic engineering because it produces a number of extracellular enzymes such as rennin, lipase, amylase and cellulase which are very important for industry (Arima, 1986, Montenecourt,1985 and Somkuti, 1968,1974). One species of Mucor, Mucor racemosus, has a haploid vegetative phase and has a small genome (10^7 bp) making it particularly convenient for genetic study. Mucor racemosus has been widely studied because it can be induced to undergo a morphogenetic change from hyphae to budding yeast-like growth in response to various environmental stimuli (Larsen, et al., 1974). A wealth of biochemical data has been generated in studies of morphogenesis, contributing to the

usefulness of Mucor racemosus as a successful model system for the study of cell growth and development.

In our laboratory, we plan to use Mucor racemosus as a model system to study the efficiency of expression and secretion of heterologous proteins from a fungus. The main goal of my research was to investigate the function of the TEF1 promoter isolated from M. racemosus. The promoter plays a very important role in gene expression because the initiation of transcription is a critical point for controlling gene expression. Finding an efficient promoter is one important step for developing an efficient heterologous protein expression and secretion system. We plan to apply the technology developed in M. racemosus to more important industrial fungi such as the closely related Zygomycetes M. miehei and M. pusillus.

HISTORICAL REVIEW

Genetic Modification of Enzymes Used in the Food Industry

Enzyme Uses in Food Areas:

Enzymes, a major group of proteins, have catalytic activities and cause a multitude of essential chemical reactions to occur at reasonable rates and at standard temperature, pressure, and reactant concentrations (Haas, 1984). Thousands of enzymes have been identified, and several dozen are important in food processing (Table 1) (Wasserman, 1988). Food processors are the largest users of industrial enzymes, especially in starch processing and cheese making (Newell, 1986). Enzymes have a long history of use in food processing, dating back to early use of calf stomachs for their rennin content to coagulate milk in cheese production. Nowadays, common uses of enzymes in the food industry include ingredient production and texture modification. Enzymes break down starch, tenderize meat, clarify wines, coagulate milk protein, and produce many other desirable changes (Potter, 1974). Some major food applications of enzymes include the production of high-fructose corn syrups, beverage clarification, brewing, baking, production of low-lactose milk, and meat tenderization (Wasserman, 1988).

Table 1. Major Enzymes Used by the Food Industry

Enzyme	Application
Proteases	
Papain, bromelain, ficin	Meat tenderization, haze removal, and chill proofing
Rennin (chymosin)	Cheesemaking
Glycosidases	
Amylases (α-, β-, gluco-, and debranching)	Baking, brewing, sweetener production
Cellulases/xylanases	Biomass conversion, juice clarification
Glucanases	Brewing
Glucose isomerase	High-fructose corn syrup (HFCS) production
Glucose oxidase/catalase	Desugaring of egg whites, oxygen removal
Glycolytic enzymes	Fermentation-carbon dioxide and ethanol production
Invertase	Candymaking
Lactase (β-galactosidase)	Low-lactose dairy products, whey disposal
Pectinases	Beverage clarification, texture modification
Lipases	
Acyl glyceride hydrolases and phospholipases	Texture modification, flavor generation

(Wasserman, 1988)

From an economic standpoint, approximately $ 160 million worth of enzymes are consumed annually by the food and detergent industries (OTA, 1981). However, only relatively few enzymes such as amylase, glucose oxidase, lipase, cellulase, protease, glucose isomerase, and invertase (Wasserman, 1984) are used in large quantities in food processing. The limited use of enzymes is related to low enzyme production and high enzyme purification cost. In order to overcome this limitation, it is required to develop economical new enzymes. Genetic engineering provides powerful tools to reach this goal.

Conventional Sources of Enzymes:

Enzymes are derived from animal, plant and microbial sources. Currently, the majority of the food processing enzymes and additives are produced by biological process (Lin, 1986). Enzymes obtained from animals are relatively expensive, and their availability usually depends on the market for those animals. For example, rennin, which is extracted from the stomach of calves, depends on the demand for lamb or beef for its availability (Loffler, 1986). Most of the commercial plant enzymes such as papain, bromelain, and ficin are easy to extract, but their supply is also governed by food demands for papaya, pineapple, and figs (Loffler, 1986). Supplies of enzymes produced by animals or

plants may fluctuate because of environmental, political, or economic factors (Haas,1984).

For technical and economic reasons, microorganisms may be the best sources for enzymes. The main reason for the attractiveness of microorganisms as potential sources of enzymes is the ease with which enzyme yields may be increased by environmental and genetic manipulations. Microbial enzymes are produced by methods that are cheap and relatively easy to scale up. Microorganisms have a rapid doubling time, which allows their production to be adjusted more easily to market demands. Environmental conditions can also be monitored closely to provide a high level of consistency in enzyme purity, stability, and activity (Loffler, 1986). Microorganisms - bacteria, yeasts, and fungi - are all used extensively in various aspects of food processing. For instance, a number of filamentous fungi (e.g. Rhizopus chinensis and Mucor miehei) secrete aspartyl proteases (rennin) which are structurally related to each other and to the gastric aspartyl protease from calf stomach (Gray et al., 1986).

In spite of the many advantages of using microbial enzymes, animal and plant enzymes are still preferred for many food processes. For example, papain, ficin, and bromelain are preferred for the chill-proofing of beer and

tenderization of meat; rennet and pepsin are the proteases of choice for the cheese industry, and plastein reactions have almost exclusively been carried out with papain, trypsin, chymotrypsin, and pepsin (Loffler, 1986). Ideally, we would like to combine the production characteristics of microbial cells (high yields, reproducibility, controllable conditions, etc.) with the use of well-known, generally recognized as safe (GRAS), plant and animal enzymes. This is now possible via recombinant DNA technology.

Enzymes production via Recombinant DNA Technology:

The single greatest obstacle limiting new enzyme development is high enzyme cost. To reduce the expense of producing and using enzymes, three broad research areas have been established: microbial strain improvement, enzyme and cell immobilization, and enzyme stability enhancement (Wasserman, 1984). Historically, microbial strain improvement has been obtained in three ways: 1) the detection, within a population, of those individuals which have undergone desirable changes; this is an inefficient, slow process; 2) the enhancement of the nucleotide sequence of DNA and the screening of the treated organisms for variants displaying the desired trait; and 3) the discovery of new organisms which naturally possess the desired quality (Haas, 1984). Significant contributions have been achieved

with these methods. However, they are time-consuming and inefficient because the mutation event is generally random across the entire genome. Since it is extremely difficult to design a random mutagenesis which generates a high proportion of the desired type of mutant, the examination of thousands of organisms is often required. This observation was the stimulus for the development of genetic engineering and related technologies.

The elucidation of the structure of DNA and the understanding of DNA replication and protein synthesis, together with the discovery of plasmids and restriction enzymes, have now made it possible to genetically engineer microorganisms (and higher organisms) to produce biochemicals which are foreign to the microorganisms. A major technique used in genetic engineering is recombinant DNA, which is the joining of pieces of DNA from different organisms to produce a hybrid molecule. This technique could theoretically allow microorganisms to express high levels of any human, animal, or plant proteins by the transfer of the genes into microorganisms. Recombinant DNA technology has essentially widened the range of enzymes that can be considered for commercialization to include even those from unusual organisms, plants, and animals (Pitcher, 1986). In some cases, yields can be increased by inserting multiple copies of the desired gene into the host organism.

Secretion systems are also being developed to allow high production levels of extracellular enzymes, a necessity in many cases to achieve favorable economics by reducing purification costs. Production of unwanted proteins or other materials can be eliminated either by specific deletion or by use of a host organism without the disadvantages of the original organism.

Recombinant DNA technology has been applied to bacteria and fungi and has contributed to the development of strong fermentation technologies in the pharmaceutical, food, and brewing industries (Haas, 1984). In 1977 it was shown that a gene from a higher eukaryote could be expressed in a microorganism, *E. coli*, to produce a biologically active protein, somatostatin (Itekura et al., 1977). Numerous mammalian gene products, including antiviral, antitumor, and antidiabetic agents and growth-promoting factors, have been expressed in microbial cells (Lin, 1986).

For enzymes in the food industries, the application of recombinant DNA technology is beginning to play a significant part in the corporate commercial product development process. As shown in Table 2, almost all the food processing enzymes and additives have been targeted by recombinant DNA techniques (Lin, 1986). Genes encoding calf rennin (Nishimori et al., 1981), thaumatin (Edens et al.,

Table 2. Food Processing Enzymes and Food Additives Used in the U.S. Food Industry that Benefit from Genetic Engineering Technology

Category	Example
Food Processing Enzymes	
Starch processing	α-Amylase β-Amylase Glucoamylase Glucose isomerase Pullulanase
Dairy products	Rennin Lipase Lactase
Brewing	Amylases Proteases
Wine/fruit/vegetable processing	Pectinases
Fuel alcohol	Amylases Glucoamylase
Food Additives	
Low-calorie products	Aspartame Thaumatin
Flavor enhancers	Glutamic acid 5'-Ribonucleotides
Human and animal diet supplements	Amino acids Vitamins
Stabilizing agents	Xanthan gum
Preservatives	Cecropin

(Lin, 1986)

1982; 1984), and cecropin(Hofsten et al.,1985) have been cloned and produced in suitable hosts, and the commercial production processes are currently under aggressive development (Lin, 1986). For example, rennin has received the most attention as a candidate for the commercialization of recombinant DNA technology in the food processing industry (Pitcher, 1986). The gene for calf rennin has been isolated and cloned into yeast (Mellor et al., 1983; Moir et al., 1985) and fungi (Cullen et al., 1987). At Genencor, much effort has been focused on the filamentous fungi for producing commercial rennin. Properly folded, active prochymosin has been secreted from Aspergillus nidulans, Aspergillus awamori, and Trichoderma reesei (Pitcher, 1986). In the last few years, at least 19 cellulase or amylase gene have been molecularly cloned (Pitcher, 1986). Many of these have been expressed in heterologous microorganisms, such as Kluyveromyces fragilis and Saccharomyces cerevisiae (Pitcher, 1986).

Another example is the lactose utilization genes which have been cloned into Saccharomyces cerevisiae (Sreekrishna and Dickson, 1985; Farahnak et al., 1986), Xanthomonas camperstris (Walsh et al., 1984), and other microorganisms so that the lactose in whey permeate can be converted into ethanol, single-cell protein, or xanthan gum. Recently Bacillus subtilis (GRAS) was utilized to produce a

thermostable α-amylase by expression of the gene from Bacillus stearothermophilus (Wasserman, 1984). This enzyme has been accepted for use in the food industry opening the way for production of other food grade enzymes via recombinant DNA technology.

The benefits of achieving commercially useful processing enzymes through genetic engineering are expected to include: 1) cost savings in enzyme production; 2) production of enzymes in GRAS organisms suitable for food processing; and 3) specific genetic modifications at the DNA level to improve enzyme properties such as thermal stability, ability to operate over a wide pH range and other performance characteristics (White, et al., 1984).

Heterologous Gene Expression Systems

Heterologous gene expression begins with the cloning of the gene(s) of interest and transfer of this genetic material to a suitable host organism. This procedure allows the investigation of the genetic activity of the DNA fragment in a characterized genetic background, the production of sufficient quantities of DNA for nucleotide sequence analysis, and the isolation of significant amount of the proteins encoded by the DNA.

The suitability of selected organisms for expression of DNA from other sources has been enhanced by the isolation of variants with advantageous mutations. For example, one strain of E. coli has been isolated which lacks a normal complement of DNA-degrading enzymes. These bacteria accept foreign DNA more efficiently than their wild-type progenitors and are routinely used as DNA recipients (Haas, 1984). The choice of the proper host organism may greatly increase the production levels of the desired protein and, perhaps even more important, high secretion levels. For instance, bacterial secretion of calf rennin has been difficult to achieve, but scientists have succeeded in secreting properly processed prochymosin, a precursor of calf rennin, from filamentous fungi (Pitcher, 1986).

E. coli as a host:

The bacterium E. coli is the most thoroughly characterized microorganism and has been the most frequently used in recombinant DNA studies. The advantages of using E. coli are the rapid growth rate of cells and the high synthesis rate of the heterologous protein. Moreover, in E. coli, several strong promoters, such as lac, lambda P_L, lambda P_R, lambda O_R/P_L, ara B, tac (combination of trp and lac), or combinations of trp, lambda P_R, and lac, have been commonly used for overproduction of enzymes and food

additive products (Lin, 1986). Until now, the most widely used host for heterologous genes has been E. coli.

In the past few years, achieving a high-level expression of foreign proteins in E. coli using various expression vectors has become a common practice. For example, rennin was produced in E. coli via recombinant DNA technology. A number of mammalian secretory proteins have been correctly processed and secreted by E. coli cells using either a natural mammalian or an E. coli signal sequence (Chang, 1987). These include rat proinsulin, human proinsulin, human immunoglobulin light chain, hGH and human epidermal growth factor (Chang, 1987).

Although a high level expression of foreign proteins in E. coli has been achieved, eukaryotic proteins expressed in E. coli are generally insoluble products. The reason is that E. coli does not carry out post-translational modifications which are necessary for activity for many enzymes, therefore the proteins produced from eukaryotic genes in E. coli may differ from the normal gene product and lack the required biological action or be insoluble until appropriately modified chemically (e.g. calf prochymosin; interferon-gamma) (Kingsman et al., 1985). For example, the rennin produced in E. coli is not secreted and forms refractile bodies of improperly folded protein. These

bodies must be solubilized and refolded to allow formation of the proper disulfide bonds. This process is expensive and, under the most practical conditions, yields are relatively poor (Pitcher, 1986). Human growth hormone (Goeddel etal., 1979) and human leucocyte α-interferon (De Maeyer et al., 1982) are produced as soluble proteins to a certain extent, but there is no indication that these products are entirely soluble. The use of prokaryotic or eukaryotic signal sequences has increased the secretion level of heterologous protein, but the level of secreted protein is generally low (Chang, 1987). E. coli also produces endotoxins and is therefore not always the preferred organism for recombinant DNA experiments with genes of interest to the food industry (Haas, 1984). Efforts are underway to apply recombinant DNA methods to organisms accepted as safe by this industry.

Bacillus subtilis as a host:

Bacillus subtilis has the potential for becoming an extremely useful system for the cloning and expression of heterologous genes. The practical advantages of this organism include its ability to secrete proteins into the medium, its non-pathogenic nature to humans, its ability to grow at relatively high temperatures, its relatively heat-stable proteins, its ability to grow on either simple or

complex growth media, and its historical use in the production of many industrial and food products (Doi, 1984).

Transformation has been employed to introduce foreign genes into B. subtilis and to alter the rate of production of heterologous enzymes. Palva (1982) cloned the B. amyloliquefaciens α-amylase into B. subtilis by a shotgun method employing the plasmid pUB110. The amylase activity released into the extracellular fluid represented a 2,500-fold increase over the activity secreted by the untransformed host (Wasserman, 1984). Through a combination of mutation and transformation, Hitotsyanagi et al. (1979), developed a strain of B. subtilis which secreted 1,500-2,000 times greater α-amylase activity than the parent.

The major problems with the B. subtilis system are the inability to carry out posttranslational modification that eukaryotic proteins seem to require to be fully functional (Van Brunt, 1986) and the low quantities of the protein product. So other host systems are being pursued.

Yeast as a host:

The yeast, Saccharomyces cerevisiae, is one of the most useful eukaryotic organisms for the study of the regulation of gene expression. Because of its small genome (only four

times the size of E. coli) and short generation time (a few hours), yeast can be experimentally manipulated as easily as most prokaryotes (Watson, 1983).

Yeast are often considered superior to E. coli as expression hosts because they can glycosylate, acetylate, fold and perform many of the other post-translational modifications that eukaryotic proteins seem to require to be fully functional (Van Brunt, 1986). Many of the eukaryotic proteins which are formed by E. coli in an inactive state are produced as soluble, biologically active proteins in S. cerevisiae (e.g. calf prochymosin, Mellor et al., 1983; IFN-gamma, Derynck, Singh and Goeddel, 1983). S. cerevisiae has an additional advantage over E. coli in that it has a secretion system which is similar to that found in higher eukaryotic organisms (Schekman and Novick, 1982) and which can be manipulated to allow the secretion of heterologous proteins. Unlike E. coli, yeast produces no endotoxins, therefore enzymes made in yeast may be more acceptable as human or animal pharmaceutical and food products (Mellor et al., 1983).

Recent studies have shown that heterologous eukaryotic genes can be expressed in the yeast S. cerevisiae when fused to specific yeast expression signals. For example, rennin (Mellor et al., 1983), glucoamylase (Innis et al., 1985),

several α-interferon genes (Hitzeman et al., 1981; Tuite et al., 1982), gamma-interferon (Derynck et al., 1983), the hepatitis B surface antigen (Valenzuela et al., 1982; Miyanohara et al., 1983), superoxide dismutase (SOD), and epidermal growth factor (EGF) (Van Brunt, 1986) have been expressed in yeast.

The study of heterologous expression in S. cerevisiae, detected several problems. Although several S. cerevisiae genes have introns (e.g. actin, ribosomal proteins) heterologous introns are not reliably processed (Langford et al., 1983). Yeast cells appear to be very stringent about the sequence specificity for recognition of splice junctions (Dobson et al., 1982). In order to express any heterologous coding sequences it is therefore important to use a cDNA. The ability of S. cerevisiae to recognize transcriptional signals in heterologous DNA is variable. In many cases, as with rabbit β-globin, there is some transcriptional activity but the mRNA initiates at the wrong site in S. cerevisiae (Kingsman et al., 1985). Experimental transfer of the rat growth hormone gene into yeast (Hitzeman et al., 1981 and Ammerer et al., 1981) showed that heterologous genes are not always accurately transcribed or properly translated. Although the secretion system of yeast cells is very similar to that in higher eukaryotic cells, the secretion level is not high even with the new "super secretor" mutants (Van

Brunt, 1986). These inherent problems of S. cerevisiae led researchers to study the filamentous fungi.

Filamentous fungi as a host:

The filamentous fungi have several characteristics which make them candidates for useful expression systems. Many filamentous fungi secrete large quantities of proteins into the culture medium. For example, certain Aspergillus niger strains secrete over five grams of glucoamylase per liter of growth medium when grown under appropriate conditions (Cullen et al., 1987). Moreover, filamentous fungi have the ability to carry out the correct posttranslational modification on many eukaryotic proteins, including the proper glycosylation pattern. Many fungal genes and genes of higher eukaryotes can also be transcribed and their transcripts processed correctly without adding specific transcription regulatory signals (Van Brunt, 1986). The technology for gene expression in filamentous fungi is developing rapidly, building on the frame-work already established for yeast.

A few examples of heterologous gene expression in filamentous fungi have been described - including the expression and secretion of M. miehei protease in A. niger, Neurospora crassa, Ustilago maydis, Phycomyces blakesleeanus

and Cochliobolus heterostrophus (Dickinson et al., 1987). Research also has been done in filamentous fungi such as production of human interferon in Aspergillus, yeast invertase in Pichia, calf rennin in Mucor, and cellulase in Trichoderma (Enari and Paavola, 1987). The secretion of chymosin by A. nidulans represents a dramatic improvement over other microbial system such as yeast and E. coli that have been used for this purpose. In A. nidulans cells transformed with the chymosin genes more than 90% of the chymosin synthesized was secreted into the culture medium and the majority of this material was enzymatically active (Cullen et al.,1987).

The filamentous fungus Mucor is an excellent target for genetic engineering since it produces many extracellular enzymes, and it provides a useful system for the study of the biochemical basis of morphogenesis. In our laboratory, we use M. racemosus as a model system to study heterologous proteins expression and secretion in a filamentous fungus.

Transformation System

The optimal use of recombinant DNA technology in the genetic study of any organism requires the availability of an efficient genetic transformation system. The technique

of DNA transformation - more particularly, plasmid-mediated transformation - presents an opportunity to transfer selected DNA molecules (genes) of interest into the host cells.

In transformation, the vector acts as a carrier to carry the genes of interest into a suitable host. Vectors are designed with particular marker genes which allow the foreign genes to be detected in the host, and ensure replication of the foreign DNA. The ability to detect the successful uptake of exogenous DNA depends on the presence of the genetic marker. One common procedure for fungal transformation is to use an auxotrophic host strain and incorporate the corresponding wild-type gene on the plasmid vector which can therefore be selected against the background of non-transformed auxotrophs (Kingsman et al., 1985). Any wild-type gene encoding an essential biosynthetic enzyme is useful as a selectable marker in cells bearing a mutation in this gene. Another useful procedure is to use dominant selectable markers whose presence can be selected for in wild-type cells (Rine and Carlson, 1986). This method is widely used in transforming bacteria with antibiotic resistance genes, especially strains which lack suitable auxotrophic mutations.

Transformation of Bacterial Cells:

Procedures for transformation and expression in some simple bacteria such as E. coli or Bacillus species and unicellular fungi such as yeast are now well established (Haas et al., 1984, Davis et al., 1980).

Transformation of S. cerevisiae:

Yeast cells are not naturally competent for DNA uptake because the cell wall is an effective barrier. This permeability barrier must be modified for DNA uptake to occur, usually by enzymatic treatment and the addition of calcium or lithium salts (Kingsman et al., 1985). These transformation systems involve enzymatic removal of the cell wall to produce sphaeroplasts which can take up DNA on treatment with polyethylene glycol and calcium ions (Kingsman et al., 1985). Under appropriate conditions the cell walls can regenerate to permit propagation and selection of transformants. Another popular method of transformation relies upon the observation that intact yeast cells treated with Li^+ ions will take up exogenous DNA (Rine and Carlson, 1985). Once the DNA enters the yeast cell, it may become integrated into one of the chromosomes or it may remain in an autonomous state, depending on the nature of the DNA molecules used in transformation (Boguslawski,

1985).

The LEU2 and TRP1 genes which encode 3-isopropylmalate dehydrogenase and N (5'- phosphoribosyl) anthranilate isomerase respectively, are commonly used as selectable markers in S. cerevisiae (Kingsman, 1985). In addition, various dominant selectable markers which confer resistance to drugs are available, e.g. thymidine kinase, chloramphenicol acetyltransferase, dihydrofolate reductase, etc (Kingsman, 1985).

Transformation of Filamentous fungi:

Filamentous fungi in general have relatively low transformation efficiencies when compared with E. coli or yeast. This is possibly due in part to an increased number of chromosomes and quantity of DNA, and to more highly regulated mechanisms of transcription and translation (Wasserman, 1988).

Protoplasts formation and regeneration of mycelium is a critical protocol for transformation of filamentous fungi. The commercially available enzyme Novozyme 234 is most convenient for this purpose by removing the cell wall. Among the filamentous fungi, the first to be transformed were a few ascomycetous species using recombinant plasmid

DNA. The transformation of Neurospora crassa was accomplished by Case et al. (1979), while the transformation of Podospora anserina and Aspergillus nidulans were first described by Stahl et al. (1982), and Ballance et al. (1983).

The genetic analysis of Mucor species is hampered by the difficulties in utilizing the sexual cycle of these organisms. Mating of (+) and (-) strains does not always result in the formation of zygospores. In some species the number of spores produced is low while in other species zygospore development remains incomplete (Schipper, 1978). Moreover, the successful germination of zygospores under laboratory conditions is rare and is preceded by a considerable period of dormancy (Van Heeswijck, 1984). These characteristics of Mucor make a transformation system desirable.

The transformation system in Mucor reported by Van Heeswijck (1984) is currently one of the most successful systems which have been developed in fungi. A high frequency transformation system was developed in this organism using protoplasts of a leucine auxotrophic mutant, M. racemosus R7B, and a shuttle vector, pLeu4, which is able to propagate in bacterial cells and in M. racemosus (Van Heeswijck, 1984). In our laboratory, we used derivatives of

pLeu4 to transform plasmid DNA into M. racemosus R7B cells.

Mucor Heterologous Protein Expression/Secretion System:

Mucor is a filamentous fungus of the class Zygomycetes which is of biological and industrial interest because of the production of a variety of extracellular enzymes and the ability of some species to undergo a morphogenetic change from hyphae to budding yeast-like growth in response to various environmental stimuli. One species, Mucor racemosus, has been widely studied because of its ability to undergo cellular morphogenesis. These studies resulted in the collection of a large quantity of biochemical research data which contributed to our choice of M. racemosus as a good model heterologous protein expression system. In our laboratory, we used M. racemosus R7B (leucine auxotroph) as a host to receive heterologous genes. The transformation vector, pLeu4, is a shuttle vector that contains a putative M. racemosus ARS (autonomous replication sequence), an E. coli replication origin, and selectable markers for E. coli (ampicillin resistance gene) and Mucor (Leu1, leucine biosynthetic gene).

In the study of gene regulation, it frequently is useful to join the promoter and controlling elements of one

gene to the structural part of another well-characterized reporter gene whose product is easy to assay. The promoter selected for use in our system is the Mucor TEF1 promoter. TEF1 is a constitutive, highly expressed gene encoding elongation factor 1α, one of the most abundant proteins present in M.racemosus (approximately 1-5 % of total protein). Because of the high level of transcription of this gene, we hypothesized that the promoter of TEF1 would be a very strong promoter (Linz et al., 1986) and useful to express heterologous genes in Mucor. To test this hypothesis, plasmids were constructed by fusing a 1.9 Kb EcoRI/HpaI DNA fragment containing the TEF1 promoter region with the lacZ gene of E. coli. These plasmids were named pMF25 and pMF29 and were transformed into Mucor cells. The level of β-galactosidase activity from the lacZ gene would indirectly measure the level of transcription driven by the TEF1 promoter. Southern analysis, Northern analysis, S-1 nuclease mapping and β-galactosidase assays showed that M. racemosus was successfully transformed with the fusion plasmids but the level of the fusion transcript detected in Northern analysis was extremely low possibly due to mRNA instability. One possible explanation was that no transcription termination signal was included in these constructs.

Since the lacZ gene proved unsatisfactory as a reporter gene to measure TEF1 promoter activity, we decided to generate a TEF1/hGH (human growth hormone) gene fusion (pMYY1) to detect the function of the TEF1 promoter. The advantages of using the hGH gene in Mucor (Chang, et al., 1987 and Selden, et al., 1986) instead of the lacZ gene are: 1) It produces stable mRNA in many eukaryotic cell types; 2) It has its own polyadenlylation site (transcription termination signal); 3) Its splice junctions are similar to those in Mucor; 4) It contains a signal peptide potentially allowing secretion from the fungus; 5) Southern and Northern analyses conducted previously showed there is no similar gene or transcript in Mucor (Wang, S.Y. 1988. unpublished); 6) Polyclonal antibody is available commercially for use in Western analysis. Expression of the hGH gene provides an excellent model system for the study of the evolution of related genes and host specific gene expression. The goal for this research was to construct a plasmid containing the TEF1/hGH gene fusion and to transform the resulting recombinant DNA into M. racemosus R7B cells. The level of transcription of this gene fusion would be measured to detect the function of TEF1 promoter.

MATERIALS AND METHODS

Microbial Strains:

Escherichia coli strain DH5α (BRL), genotype: F⁻phi 80dlacZΔM15Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r_K^-, m_K^+)supE44lambda⁻thi-1 gyrA relA1, was modified from the parental strain DH5 (Hanahan, 1983). The strain was used for amplification and isolation of plasmid DNA (pMR1-3, pOGH and pMYY-1) and also as the recipient strain in bacterial transformation experiments. This bacterial strain is sensitive to ampicillin, so plasmid encoded ampicillin resistance can be used to screen transformants.

Mucor racemosus ATCC 1216b R7B, a leucine auxotroph, was derived from Mucor circinelloides f. lusitanicus CBS 277.49 (Syn. Mucor racemosus ATCC 1216b) (VanHeeswijck, 1984). It was used as the recipient strain in the Mucor transformation experiment.

Plasmids:

The plasmid pLeu4 (which was kindly donated by Carlsberg laboratory, Denmark) carries the β-lactamase gene (Amp^R) in pUC13 for selection in the ampicillin sensitive strain, E. coli DH5α, plus a 4.4 Kb PstI restriction

fragment of Mucor racemosus genomic DNA which contains a leucine biosynthetic gene as a selectable marker in Mucor racemosus ATCC 1216b R7B, (Van Heeswijck and Roncero, 1984). This restriction fragment also carries a putative Mucor autonomous replication sequence (ARS) (Roncero et al., 1988) (Fig. 1, pp.31, 32).

The plasmid pMR1-3 was derived from pLeu4 by inserting a 1.9 Kb EcoRI-HpaI fragment from TEF-1 containing a putative strong promoter (Fig. 2, pp.33, 34).

The plasmid p0GH, donated by R.Selden (Harvard Medical School), is a pUC19 based vector which contains a promoterless gene encoding human growth hormone (hGH) (Fig. 3, pp.35, 36).

Chemicals:

Most chemicals used in the experiments were purchased from Sigma chemical company with the exception of those described specifically in the text. Restriction endonucleases, T4 DNA ligase, DNA polymeraseI (Klenow fragment), 2'-deoxynucleotides, and the buffers utilized in DNA manipulations were obtained from Boehringer Mannheim Biochemicals. (α-^{32}P)dGTP (>6000 Ci/mmole) was purchased from New England Nuclear (USA). Polyethylene glycol 4000

Figure 1. Restriction map of plasmid pLeu4.

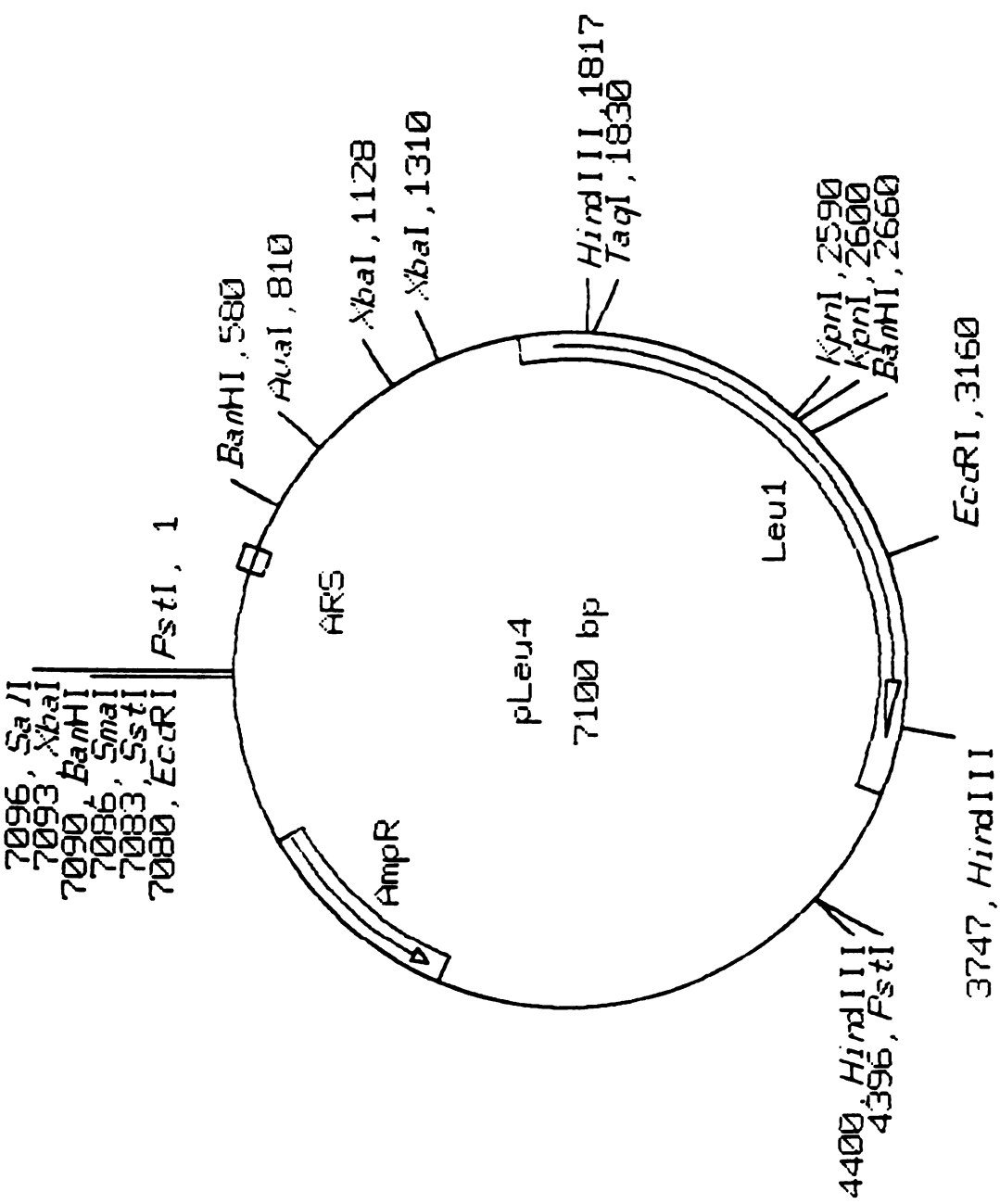

7096, Sal I
7093, XbaI
7090, BamHI
7086, SmaI
7083, SstI
7080, EcoRI
FstI, 1
BamHI, 580
AvaI, 810
XbaI, 1128
XbaI, 1310
HindIII, 1817
TaqI, 1830
KpnI, 2590
KpnI, 2600
BamHI, 2660
EcoRI, 3160
3747, HindIII
4400, HindIII
4396, FstI
ARS
AmpR
pLeu4
7100 bp
Leu1

Figure 2. Restriction map of plasmid pMR1-3.

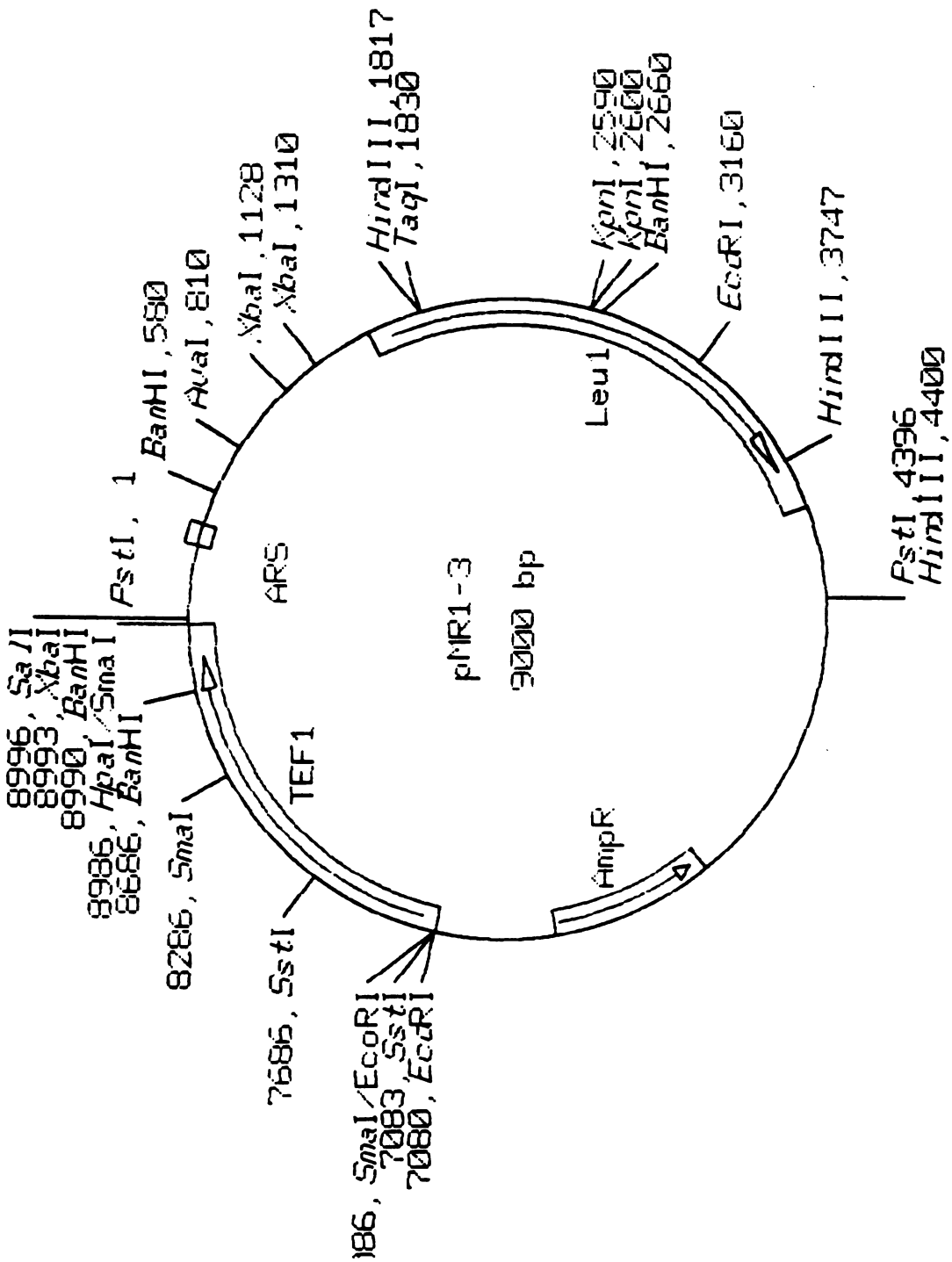
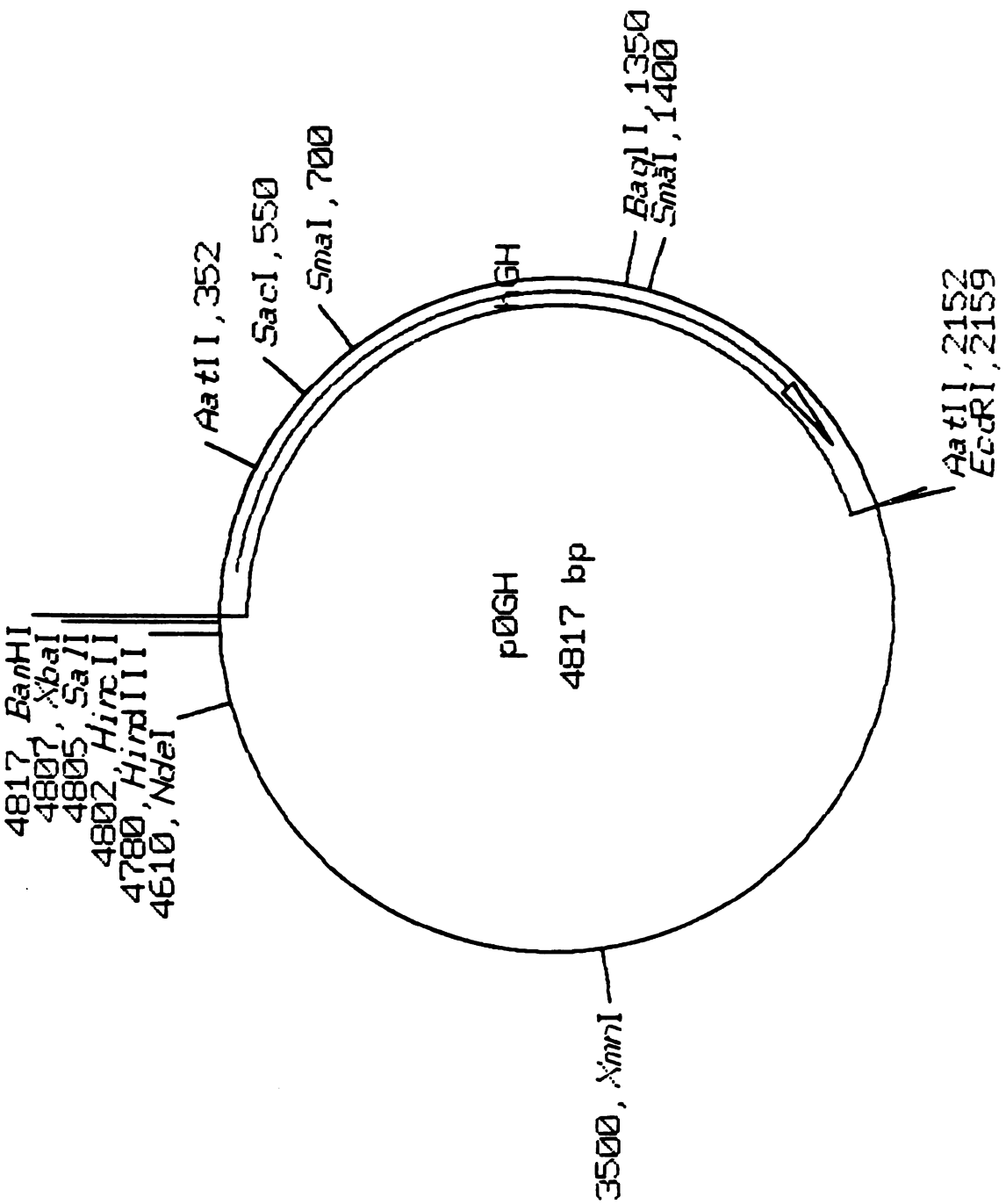

pMR1-3
9000 bp
ARS
TEF1
Leu1
AmpR
PstI, 1
BamHI, 580
AvaI, 810
XbaI, 1128
XbaI, 1310
HindIII, 1817
TaqI, 1830
KpnI, 2590
KpnI, 2600
BamHI, 2660
EcoRI, 3160
HindIII, 3747
PstI, 4396
HindIII, 4400
186, SmaI/EcoRI
7083, SstI
7080, EcoRI
7585, SstI
8286, SmaI
8686, BamHI
8986, HpaI/SmaI
8990, BamHI
8993, XbaI
8996, SalI

Figure 3. Restriction map of plasmid pOGH.


4817 BamHI
4807, XbaI
4805, SalI
4802, HincII
4780, HindIII
4610, NdeI
AatII, 352
SacI, 550
SmaI, 700
BglI, 1350
SmaI, 1400
AatII, 2152
EcoRI, 2159
3500, XmnI
pØGH
4817 bp

(PEG 4000) was obtained from Fluka AG (Switzerland). Novozyme 234 (prepared from Trichoderma harzianum; mainly containing α-1,3-glucanase activity) was from Novo Industries. Crude chitosanase (isolated from Myxobacter AL-1) was prepared in our laboratory.

Growth Medium and Growth Conditions for Escherichia coli:

E. coli cells were grown at 37°C in Luria-Bertani (LB) medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 10 g NaCl per liter, pH 7.5) with constant shaking or on solid medium containing 1.5% (wt./vol.) Bacto-agar. E. coli strains containing a plasmid which carries the ampicillin resistance gene were grown on LB medium supplemented with ampicillin (50 ug/ml). YT medium (8 g Bacto-tryptone, 5 g Bacto-yeast extract and 5 g NaCl per liter, pH 7.2-7.4; 1.5% wt./vol. Bacto-agar for solid medium) was used in the bacterial transformation experiments.

Transformation of Escherichia coli:

E. coli strain DH5α was used as a host strain in bacterial transformation. Competent cells for plasmid transformation were prepared by the Calcium Chloride procedure (Maniatis et al., 1982). Calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) were used to introduce cracks

in the bacterial cell walls. A single colony of E. coli strain DH5α was inoculated into 3 ml YT broth at 37°C with shaking (ca. 225 rpm), overnight. An appropriate amount of the overnight culture was diluted 1 to 200 with YT broth (100 ml is sufficient for 20 transformation reactions) and incubated with shaking at 37°C until mid log phase (A590=0.4-0.6; ca. 2-3 hours). The cells were chilled on ice, pelleted by centrifugation (7000X g, 5 minutes at 4°C), gently resuspended in ice-cold 0.1 M MgCl2 (1/5 of the original volume) and kept on ice for 15 minutes. The cells were pelleted again by centrifugation (7000X g, 5 minutes at 4°C), gently resuspend in ice-cold 0.1 M $CaCl_2$ (1/50 of the original volume) and kept on ice for 60 minutes to generate competent cells.

An aliquot of ligation mixture (5 ul ≈ 10 ng)was added to 100 ul competent cells at 4°C. The tube was shaken gently and then kept on ice for 30 minutes. The cell mixture was heated to 42°C for 2 minutes without shaking (heat shock) and then placed on ice for 2 minutes. Nine volumes (900 ul) of SOC (2% wt./vol. Bacto-tryptone, 0.5% wt./vol. Bacto-yeast extract, 10 mM NaCl 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) held at room temperature were added to the cell mixture which was then incubated by shaking at 37°C for 60 minutes. One hundred ul aliquots of transformed cells were spread onto YT medium

containing ampicillin (50 ug/ml), and incubated at 37°C for 24 hours.

Colony Hybridization for the Identification of Recombinant Plasmids in E. coli:

Transformants containing the desired recombinant plasmid were identified by the colony hybridization procedure (Maniatis T., et al, 1982, Grunstein and Hogness, 1975) using ^{32}P-labeled gene-specific DNA probes. This method was used to screen small numbers (100-200) of colonies isolated from several agar plates. Transformant colonies, which grew on selective plates, were consolidated onto a master agar plate and onto a nitrocellulose filter laid on the surface of a second agar plate. After a period of growth (ca.24 hr), the colonies on the nitrocellulose filter were lysed and the DNA denatured with Southern base (1.5 M NaCl and 0.5 M NaOH) and then neutralized with Southern neutralizer (1 M Tris-Cl and 1.5 M NaCl, pH 8.0). The DNA liberated from these colonies was fixed to the filter by baking for 2 hours at 80°C under vacuum. The master plate was stored at 4°C until the results of the screening procedure were available.

The baked filters were floated on the surface of 6X SSC solution until throughly wetted from beneath, then submerged

into the solution for 5 min. The wet filters were put into 100-300 ml prewashing solution (50 mM Tris.Cl [pH 8.0], 1 M NaCl, 1 mM EDTA and 0.1% SDS) and incubated for 2 hours at 42°C. The filters were incubated in prehybridization solution (6X SSC, 5X Denhardt's solution [Maniatis et al., 1982], 50% formamide, 0.1% SDS, 100 ug/ml denatured salmon sperm DNA , 5 mM EDTA) for 2 hours at 42°C. An appropriate amount of denaturated probe was added to the prehybridization solution, and the hybridization was performed at 42°C for 12 to 36 hours. After two non-specific washes (1X SSC, 0.1% SDS, 15 min at room temperature) and one high stringency final wash (0.1X SSC, 0.1% SDS, 1 hr at 65°C), the filters were analyzed by autoradiography. Colonies which gave positive autoradiography results were recovered from the master plates.

Isolation of Plasmid DNA from E.coli:

Plasmid DNA was obtained from small cultures of E. coli by the alkaline lysis "miniprep" method (Maniatis et al., 1982). Plasmid DNA purified in this way was treated with DNase-free RNase (20 ug/ml) for one hour at 37°C during restriction enzyme digestion.

For large-scale preparation of plasmid DNA (Maniatis et al., 1982), a small volume of overnight culture (0.1 ml) was added to 25 ml of LB medium containing ampicillin (50 ug/ml) in a 100 ml flask and incubated at 37°C with vigorous shaking until the culture reached late log phase (OD_{600} = 0.6). Twenty-five ml of the late log phase culture was inoculated into 500 ml of LB medium with ampicillin (50 ug/ml) in a 2-liter flask, incubated exactly 2.5 hr at 37°C with vigorous shaking ($OD_{600} \approx 0.4$) followed by addition of 2.5 ml chloramphenicol (34 mg/ml in ethanol) to amplify the plasmids. Alkaline lysis was performed on the cells (Maniatis et al., 1982). The plasmid DNA was purified by equilibrium centrifugation of the lysate in cesium chloride - ethidium bromide gradients. Ethidium bromide was removed from the purified plasmid DNA by isoamyl alcohol extraction followed by dialysis to remove cesium chloride. The DNA was stored in TE buffer (10 mM Tris.Cl [pH 8.0] and 1mM EDTA)at 4°C.

Growth Conditions for Mucor :

Sporangiospores of Mucor racemosus ATCC 1216b and the leucine auxotroph strain R7B were produced on YPG agar plates (0.3% yeast extract, 1% peptone and 2% glucose; the pH of medium was adjusted to 4.5 with conc. sulfuric acid) which were inoculated with a small amount of stock

sporangiospores in the center and incubated for 5-7 days at 28°C. The mature spores were harvested with ice-cold distilled water by scraping the surface of the medium with a sterile glass rod. The harvested spores were inoculated into fresh medium immediately or stored as a spore stock at -20°C in sterile distilled water containing 20% (vol/vol) glycerol.

Mucor racemosus ATCC 1216b was also grown in YNB minimal medium (0.5% Difco yeast nitrogen base [without amino acids and ammonium sulfate], 1.5% ammonium sulfate, 1.5% glutamic acid; pH was adjusted to 4.5 with conc. sulfuric acid). After steam sterilization, the medium was supplemented with 1% (w/v) sterile glucose, 1 ug/ml thiamine and 1 ug/ml niacin. Mucor racemosus ATCC 1216b R7B (leucine auxotroph) was also grown in YNB minimal medium supplemented with 1 mM leucine. Mucor racemosus R7B, transformed with plasmids pMF25, pMF29, pMR1-3 or pMYY-1, was grown on YNB minimal medium without leucine to provide constant selective pressure for the presence of the plasmids.

Transformation of Mucor:

Fresh sporangiospores of Mucor racemosus 1216b R7B were suspended in 50 ml YPG medium (at a concentration of $2x10^5$ spores/ml) and germinated about 5.5 hours at 28°C with

vigorous shaking. The germlings, whose germ tubes were 3 to 5 spore diameters in length, were harvested by filtration through nylon cloth (mesh size 30 um) and washed twice with 0.5 M sorbitol. The germlings were incubated 1 hour at 28°C with mild shaking in 1 ml 0.01 M sodium phosphate buffer (pH 6.5) containing 0.5 M sorbitol, Chitosanase (200 units/ml) and Novozyme (2 mg/ml) to produce protoplasts.

The protoplasts and undigested cells were harvested by centrifugation (400X g, 8 minutes at room temperature), washed twice with 0.5 M sorbitol, washed once with 0.5 M sorbitol in Mops(3-N-Morpholine propane sulphonic acid) buffer (10 mM Mops pH 6.3, 50 mM $CaCl_2$), and then centrifuged (400X g, 8 minutes at room temperature) to harvest the protoplasts. The cell pellet was suspended in 2 ml of 0.5 M sorbitol in Mops buffer. The number of protoplasts was counted with a hemacytometer under a light microscope. The protoplasts were observed to burst after adding distilled water.

The plasmid mixture (containing 0.1-50 ug plasmid DNA) was pretreated with 1 mg heparin in 20 ul of 0.5 M sorbitol in Mops buffer for 20 minutes on ice and added to 0.2 ml of the protoplast suspension. A 20 ul aliquot of 40% PEG 4000 in Mops buffer was added to this reaction mixture which was incubated on ice for 30 minutes. After incubation, 2.5 ml of

40% PEG 4000 in Mops buffer was added and the incubation continued at room temperature for 25 minutes to complete the transformation. The cell suspensions were diluted with 20 ml of 0.5 M sorbitol in Mops buffer and centrifuged (400X g, 5 minutes at room temperature). The cell pellet was resuspended in 5 ml of YPG broth (pH 4.5) with 0.5 M sorbitol and incubated at room temperature for 30 minutes to allow the cells to recover. The pellet was harvested by centrifugation (400X g, 5 minutes at room temperature) and resuspended in 5 ml of YNB (pH 4.5) broth with 0.5 M sorbitol. Aliquots of these cell suspensions (0.1 ml and 1 ml) were added to a 9 ml YNB agar overlay (containing 0.5 M sorbitol, pH 3.0; 1% w/v agar) and inoculated onto YNB (containing 0.5 M sorbitol, pH 3.0) agar plates. The lower pH of the medium limited the size of transformant colonies. The plates were incubated at room temperature for 2-3 days before colonies were counted.

Single Colony Isolation of Mucor Transformants:

Sporangiospores of the putative Mucor transformants were harvested with 200ul ice-cold distilled water by scraping the surface of the mycelia of each single isolated colony with a sterile loop.

The harvested spore aliquots were diluted with YNB broth (1 to 10^{-3}) and inoculated onto YNB agar plates which were incubated in an anaerobic jar (BBL GasPak-anaerobic system) at room temperature for about 5 days to generate isolated yeast colonies. Four colonies of each transformant were picked and reinoculated onto YNB medium to produce sporangiospore stocks.

Isolation of Genomic and Plasmid DNA from Mucor:

DNA was isolated from Mucor strains as described by Van Heeswijck (1984) with the following modification. Fresh sporangiospores (10^7 spores/ml) were inoculated into 300 ml of YNB (pH 4.5) minimal broth at 28°C with constant shaking. The germinated mycelia, 12 to 15 spore diameters in length, were harvested by filtration through filter paper (Whatman 1 mm), frozen in liquid nitrogen and ground to a powder with a mortar and pestle. The ground cells were suspended in 8 ml TES buffer (100 mM Tris.Cl [pH 8.0] , 150 mM NaCl, 100 mM EDTA and 0.1% SDS) and mixed for one minute at room temperature. An equal volume of phenol equilibrated with TES buffer was added and mixed gently for 1 hour on a rocker platform. The aqueous phase was separated from the organic phase by centrifugation (3000X g, 10 min at 4°C) and the aqueous phase was recovered. This process was repeated. The aqueous phase was extracted with an equal volume of

phenol (equilibrated with TE buffer)/chloroform:isoamyl alcohol (25/24:1) by mixing gently for 30 min on a rocker platform. After the aqueous phase was recovered, the DNA was precipitated with two volumes of ethanol for 30 minutes at -20°C. The precipitated DNA was collected by centrifugation (12000X g, 20 min at 4°C), dried under vacuum, and dissolved in 1 ml of TE buffer. RNase (50 ug/ml) was added to the solution which was incubated for 2 hours at 37°C followed by addition of proteinase K (100 ug/ml). The incubation was continued for 2 hours at 37°C. The DNA mixture was extracted with an equal volumes of phenol (saturated with TE) and chloroform:isoamyl alcohol (25/24:1) by mixing for 5 min on a rocker platform. The aqueous phase was recovered by centrifugation (3000X g, 10 min at 4°C), and the DNA was precipitated by addition of NaOAC (final concentration is 0.25 M) and two volumes of ethanol and stored overnight at -20°C. The DNA was collected by centrifugation and vacuum dried. This DNA pellet was dissolved in TE buffer and quantitated by measuring the absorbance of the solution at 260 nm with a spectrophotometer. An OD_{260} of 1 corresponds to approximately 50 ug/ml for double-stranded DNA. A pure preparation of DNA has OD_{260}/OD_{280} of 1.8.

Restriction Endonuclease Analysis and Gel Electrophoresis of DNA:

DNA was digested with restriction enzymes according to the conditions recommended by the manufacturer. The DNA fragments were separated according to size by electrophoresis through agarose gels (0.8 to 1.5 %) together with lambda-DNA/HindIII size marker. Gels were stained with ethidium bromide (0.5 ug/ml) and photographed by transillumination with UV light.

DNA restriction fragments for use in cloning or for preparing DNA probes were purified by agarose gel electrophoresis followed by electroelution using an apparatus from International Biotechnologies, Inc. (IBI).

Preparation of RNA from Mucor:

RNA was extracted from Mucor cells by the hot phenol method described by Maramatsu (1973). Fresh sporangiospores (10^7 spores/ml) were inoculated into 300 ml of YNB (pH 4.5) minimal broth at 28°C with constant shaking. The mycelia, which were 12 to 15 spore diameters in length, were harvested by filtration through filter paper (Whatman 1mm), frozen in liquid nitrogen and ground to a powder with a mortar and pestle. The ground cells were transferred to a

cold 30 ml corex tube and suspended in 1 ml of cold SDS-RNA-X buffer (50 mM NaOAC, 1 mM EDTA, 1% SDS, pH 5.0) treated with diethyl pyrocarbonate (DEPC). Four ml of hot SDS-RNA-X buffer (65°C) was added to the solution followed immediately by 5 ml of hot phenol saturated with RNA-X buffer (65°C). The mixture was vortexed for 30 sec. The extraction mixture was incubated for 15 min at 65°C . The aqueous phase was recovered by centrifugation (4300x g, 5 min at room temperature) and reextracted with an equal volume of hot phenol saturated with RNA-X buffer. The aqueous phase was recovered and extracted with one volume of phenol and chloroform:isoamyl alcohol (25/24:1). The aqueous phase was recovered, extracted with an equal volume of chloroform : isoamyl alcohol (24:1) and then extracted two times with one volume of diethyl ether (water saturated). The upper phase (ether phase) was removed. The RNA was precipitated from the aqueous phase by addition of 1/6 volume of 3 M NaOAC and 2.5 volumes of ethanol and stored overnight at -20°C. The RNA was recovered by centrifugation (12000X g, 10 min at 4°C) and the pellet was washed with 70% ethanol. The RNA pellet was dried under vacuum and dissolved in H_2O treated with DEPC. The concentration and purity of RNA was measured with a spectrophotometer. An OD_{260} of 1 corresponds to approximately 40 ug/ml RNA. A pure preparation of RNA has OD_{260}/OD_{280} of 2 . The RNA samples were stored at -70°C in a freezer.

Gel Electrophoresis of RNA:

RNA was denatured by treating the sample with formamide/ formaldehyde and size fractionated by electrophoresis through 1% to 1.5% (w/v) formaldehyde-agarose gels (Maniatis et al., 1982, Fourney et al., 1988) with a RNase-free Mops/EDTA buffer system (10X stock solution: 0.2 M Mops [3-(N-morpholinolino)-propanesulfonic acid], 50 mM sodium acetate, 10 mM EDTA, pH 7.0; treated with diethyl pyrocarbonate (DEPC) at least 12 hr and then autoclaved). After electrophoresis, the lane containing the RNA size standards was separated from the rest of the gel and stained in a solution containing 10 mM $MgSO_4$ and 0.5 ug/ml ethidium bromide for 45 min. The excess ethidium bromide and formaldehyde were removed by soaking the gel in 1 mM $MgSO_4$ solution for 1 hour. The image of RNA ladder was photographed by transillumination with UV light.

Southern and Northern Analyses of Nucleic Acid:

Identification of similarities between sequences of DNA was accomplished by the hybridization technique described by Southern (1975). DNA fragments were separated according to size by electrophoresis through an agarose gel, denatured with Southern base, and neutralized with Southern neutralizer. The denatured DNA fragments were transferred

and immobilized to a nitrocellulose filter (BA 85, from Schleicher and Schuell FRG) using the protocol of Southern (1975) described in Maniatis (1982). A 10X SSC buffer system was used to transfer DNA by capillary action. The filter with transferred DNA was air dried and baked for 2 hours at 80°C under vacuum.

The quantities of specific RNA transcripts were analyzed by Northern analysis (Maniatis, 1982). RNA was denatured with formamide/formaldehyde and size fractionated by formaldehyde-agarose gel electrophoresis. The gel was prepared for transfer by soaking it for two 20 min periods in 10X SSC solution at room temperature with gentle shaking. The RNA was transferred to a nitrocellulose filter using the same protocol as for Southern blotting.

DNA fragments which were used as probes were labeled with α-^{32}P-dGTP by the random primer method (Feinberg, and Vogelstein, 1983). Nitrocellulose filters carrying target nucleic acid were hybridized to the denatured gene-specific ^{32}P-labeled DNA probes. Filters were prehybridized for 2 hours in prehybridization solution (6X SSC, 5X Denhardt's solution, 50% formamide, 100 ug/ml of denatured salmon sperm DNA, 0.1% SDS and 5 mM EDTA). An appropriate amount of probe (1-5 x 10^8 cpm/ml) was added and hybridizations were carried out at 42°C for the required hybridization period

(usually 16-24 hr). After hybridization, the nitrocellulose filter was washed twice in 1X SSC/0.1% SDS for 15 min and once in 0.1X SSC/0.1% SDS for 1 hr at 42°C for low stringency or 65°C for high stringency. The washed filter was air dried, sealed with Saran Wrap, and autoradiographed with Kodak x-ray film with or without an intensifying screen for the required exposure time. The position of target bands complementary to the radioactive probe was observed by soaking the film in Kodak developer for 5 min and rapid fixer for 5 min.

Nuclease-S1 Mapping of RNA:

Nuclease-S1 mapping was used to map the ends of RNA molecules and the location of any splice points in relation to specific restriction sites within the template DNA. Total RNA (100 ug) was mixed with 0.1 ug template DNA, and the nucleic acids were coprecipitated with ethanol at -70°C for 15 min. The nucleic acid pellet was resuspended in 0.03 ml of hybridization buffer (40 mM PIPES [piperazine-N, N'-bis (2-ethanesulfonic acid)] [pH 6.4], 1 mM EDTA [pH 8.0], 0.4 M NaCl and 80% formamide) and heated to 80°C for 10 min to denature the DNA. The tubes were transferred rapidly to a 49°C water bath and incubated for 3 hours. After incubation, 0.3 ml of ice-cold S1 nuclease buffer (0.28 M NaCl, 0.05 M sodium acetate [pH 4.6] and 4.5 mM $ZnSO_4$)

containing 400 units of S1 nuclease was added. The tubes were mixed well and incubated at 20°C for 30 min followed by 30 min at 4°C. The DNA/RNA hybrids resistant to S1 digestion were precipitated with 2.5 volumes of ethanol after addition of 10 ug of yeast tRNA. The precipitate was dissolved in TE buffer and resolved on a neutral agarose gel. Gels were further analyzed by Southern analysis as described above.

β-Galactosidase Assay:

Fresh sporangiospores (10^7 spores/ml) were inoculated into 300 ml of YNB (pH 4.5) broth at 28°C with constant shaking. The mycelia, 8 to 10 spore diameters in length, were harvested by filtration through a filter paper (Whatman 1 mm), frozen in liquid nitrogen and ground to a powder with a mortar and pestle. The ground cells were resuspended in 1 ml of Z buffer (per liter, 16.1 g of $Na_2HPO_4.7H_2O$, 5.5 g of $NaH_2PO_4.H_2O$, 0.75 g of KCl, 0.246 g of $MgSO_4.7H_2O$, pH adjusted to 7.0, autoclaved, cooled, then 2.7 ml of 2-mercaptoethanol was added). Three drops (pasteur pipette) of $CHCl_3$ and 2 drops of 0.1% SDS were added. The cell suspension was vortexed at high speed for 10 sec. A 0.2 ml aliquot of ONPG (o-Nitrophenyl-β-D-galactoside, 4 mg/ml solution [dissolved in H_2O]) was added to samples which were incubated at 28°C for up to 2hr. The reaction was stopped by adding 0.5 ml of

1 M Na_2CO_3, and the cell debris was removed by centrifugation (7000x g, 5 min). The enzyme activity of the reaction mixture was determined by spectrophotometry (OD_{420}). Because of the low level of β-galactosidase activity observed in extracts, the protein concentration of the extract was not determined (J.H. Miller, ed., 1972).

RESULTS

(1) TEF1/lacZ Fusion Plasmid:

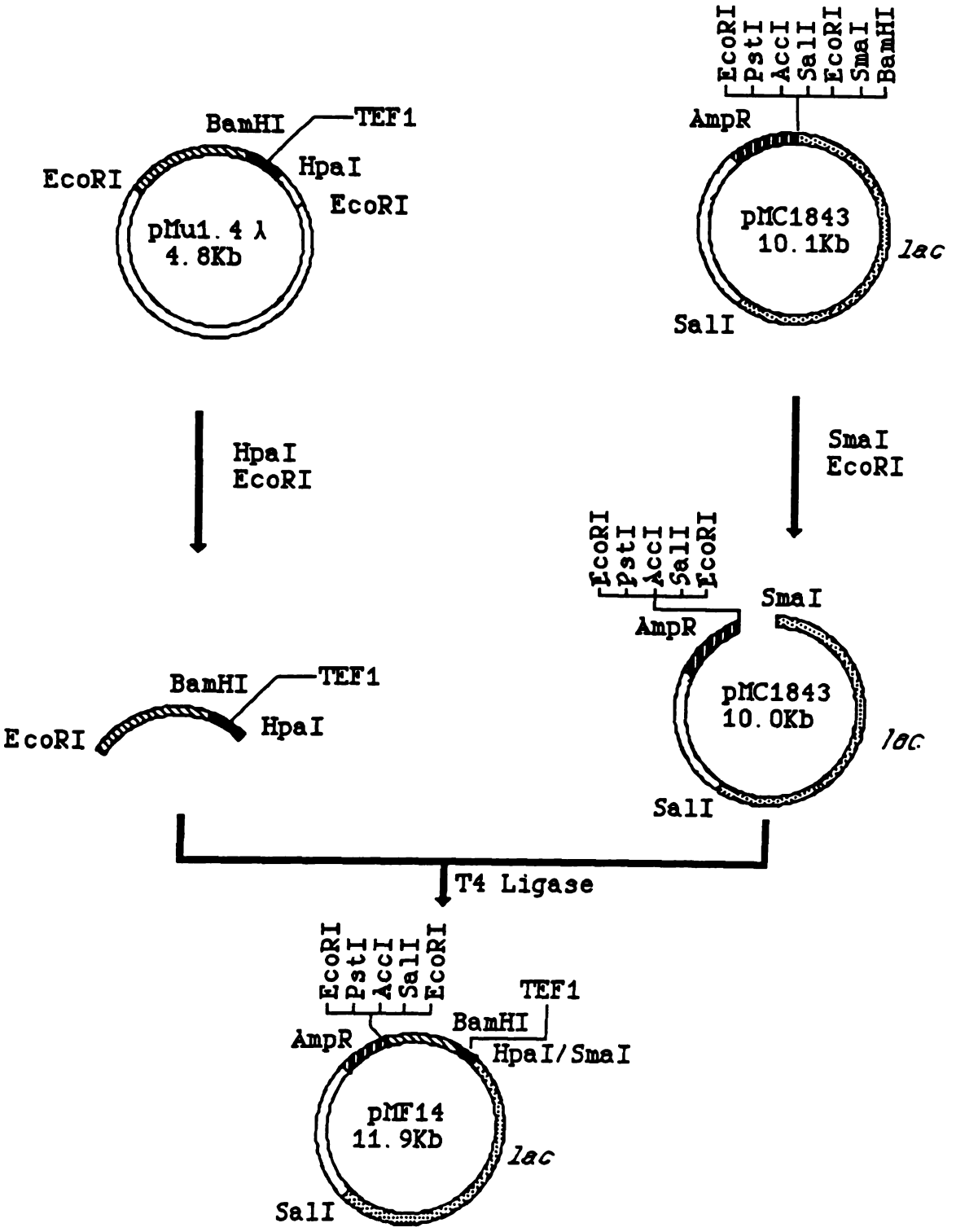
Construction of Gene Fusion Plasmids

Gene fusions were initially constructed between the Mucor TEF1 promoter and the lacZ gene of E.coli to measure the activity of this Mucor promoter. TEF1 encodes elongation factor 1α and was predicted to generate large quantities of the fusion transcript. The construction of the original fusion plasmid pMF14 is outlined in Figure 4 (pp.55. 56). Since pMF14 did not contain a selectable marker for transformation of Mucor, the 8.1 kb SalI restriction fragment containing the TEF1/lacZ gene fusion was gel purified from a restriction digest of plasmid pMF14 and ligated into the SalI site of pLeu4. Plasmids with two orientations of the TEF1/lacZ fusion in pLeu4 were obtained after ligation and called pMF25 and pMF29.

Mucor racemosus ATCC 1216b R7B was transformed with pMF25 and pMF29 in order to demonstrate the function of the TEF1 promoter and the expression of lacZ gene in Mucor cells. The transformants grew when transferred to fresh minimal media (YNB). The above work was conducted by Ms. Fumin Chiu.

Figure 4. Construction of plasmid pMF14.

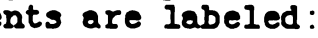
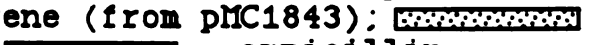
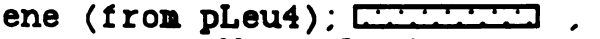
The regions representing particular DNA fragments are labeled:  , TEF1 promoter region;  , ampicillin resistance gene;  , lac operon.


BamHI
TEF1
EcoRI
HpaI
EcoRI
pMu1.4 λ
4.8Kb
HpaI
EcoRI
BamHI
TEF1
EcoRI
HpaI
EcoRI PstI AccI SalI EcoRI SmaI BamHI
AmpR
pMC1843
10.1Kb
lac
SalI
SmaI
EcoRI
EcoRI PstI AccI SalI EcoRI
SmaI
AmpR
pMC1843
10.0Kb
lac
SalI
T4 Ligase
EcoRI PstI AccI SalI EcoRI
TEF1
BamHI
AmpR
HpaI/SmaI
pMF14
11.9Kb
lac
SalI

Southern Analysis of DNA Isolated from Mucor Transformants

Genomic DNA was extracted from Mucor racemosus ATCC 1216b R7B transformed with plasmids pMF25 and pMF29. The DNA was digested to completion with the restriction endonucleases SalI and EcoRI. The EcoRI digest was performed to distinguish between the two possible orientations of TEF1/lacZ gene insert (see restriction maps, Figure 5, 6, 7, pp.58, 59, 60, 61, 62, 63). The DNA restriction fragments were separated by electrophoresis through a 0.8% agarose gel and transferred to a nitrocellulose filter. A 6.2 Kb SalI fragment containing the lacZ gene was gel purified from a SalI restriction digest of plasmid pMC1843, radioactively labeled with ^{32}P, and used to hybridize to the nitrocellulose filter for 16 hours. The filter was washed under high stringency conditions and exposed to a X-ray film. The lacZ gene probe hybridized to a 8.1 Kb SalI restriction fragment and to a 11.2 Kb EcoRI restriction fragment in genomic DNA extracted from the putative pMF25 transformant. The lacZ gene probe hybridized to a 8.1 Kb SalI fragment and to a 8.1 Kb EcoRI fragment in DNA extracted from the pMF29 transformant. The lacZ gene probe did not hybridize to DNA isolated from the nontransformed host strain or pLeu4 transformants. This data confirmed the entry of plasmids pMF25 and pMF29 into the host strain during transformation.

Figure 5. Construction of plasmids pMF25 and pMF29.

The regions representing particular DNA fragments are labeled:  , TEF1 promoter region;  , ampicillin resistance gene (from pMC1843);  , lac operon;  , ampicillin resistance gene (from pLeu4);  , *Mucor* ARS;  , *Mucor* Leu1.

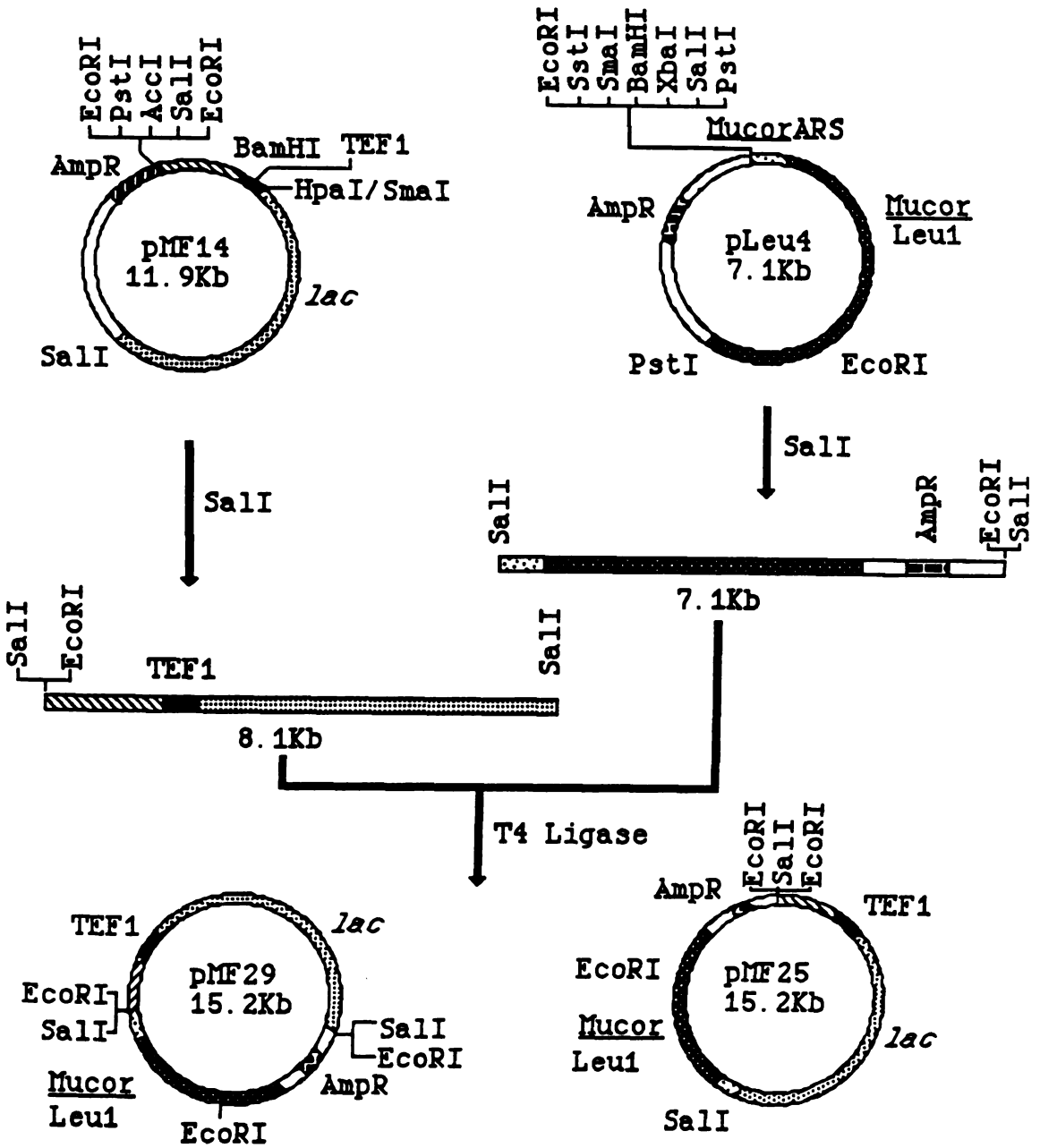

EcoRI
PstI
AccI
SalI
EcoRI
AmpR
BamHI
TEF1
HpaI/SmaI
pMF14
11.9Kb
lac
SalI
EcoRI
SstI
SmaI
BamHI
XbaI
SalI
PstI
MucorARS
AmpR
pLeu4
7.1Kb
Mucor
Leu1
PstI
EcoRI
SalI
SalI
AmpR
EcoRI
SalI
7.1Kb
SalI
EcoRI
TEF1
SalI
8.1Kb
T4 Ligase
TEF1
lac
pMF29
15.2Kb
EcoRI
SalI
SalI
EcoRI
Mucor
Leu1
AmpR
EcoRI
AmpR
EcoRI
SalI
EcoRI
TEF1
EcoRI
pMF25
15.2Kb
Mucor
Leu1
lac
SalI

Figure 6. Restriction map of plasmid pMF25.

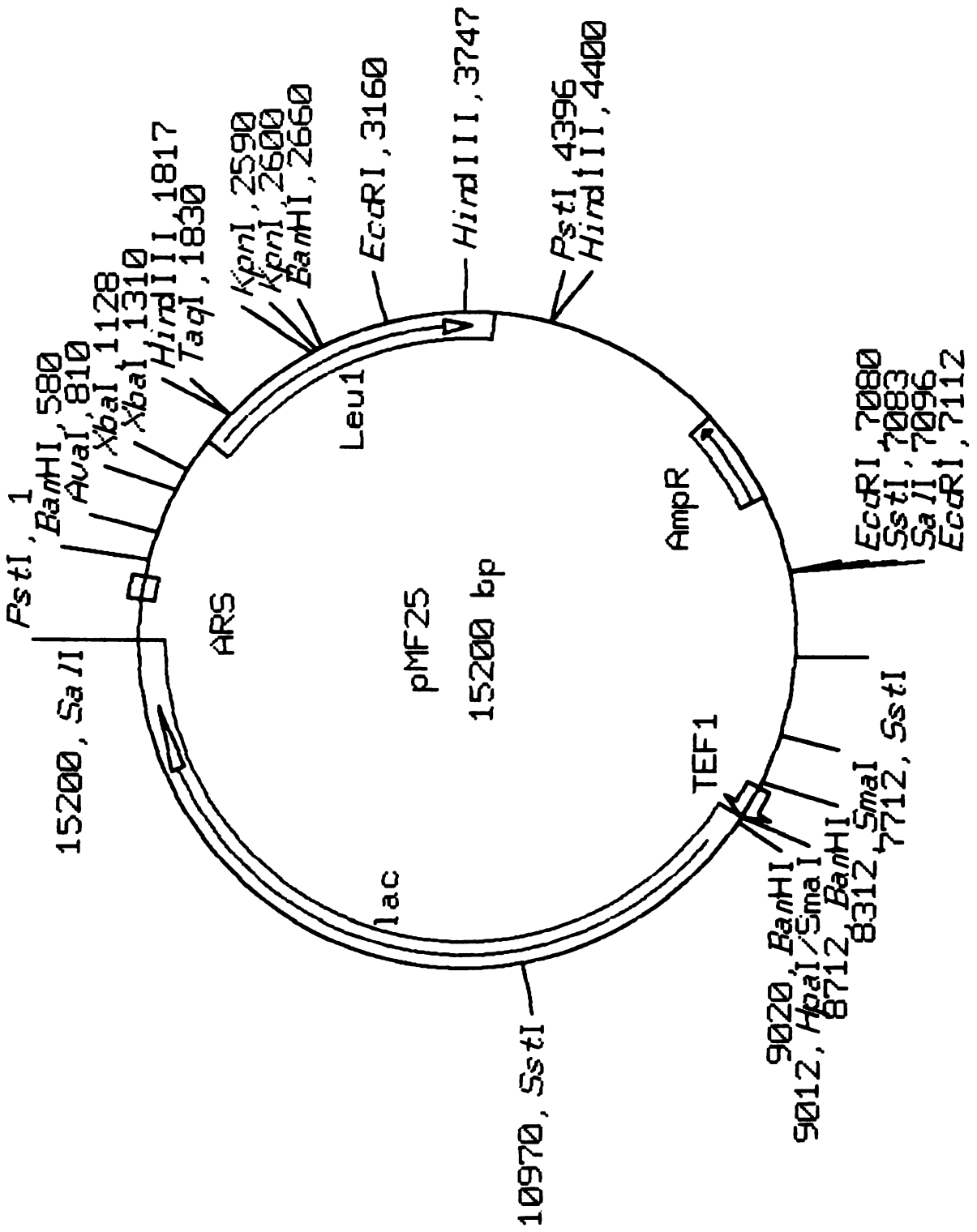
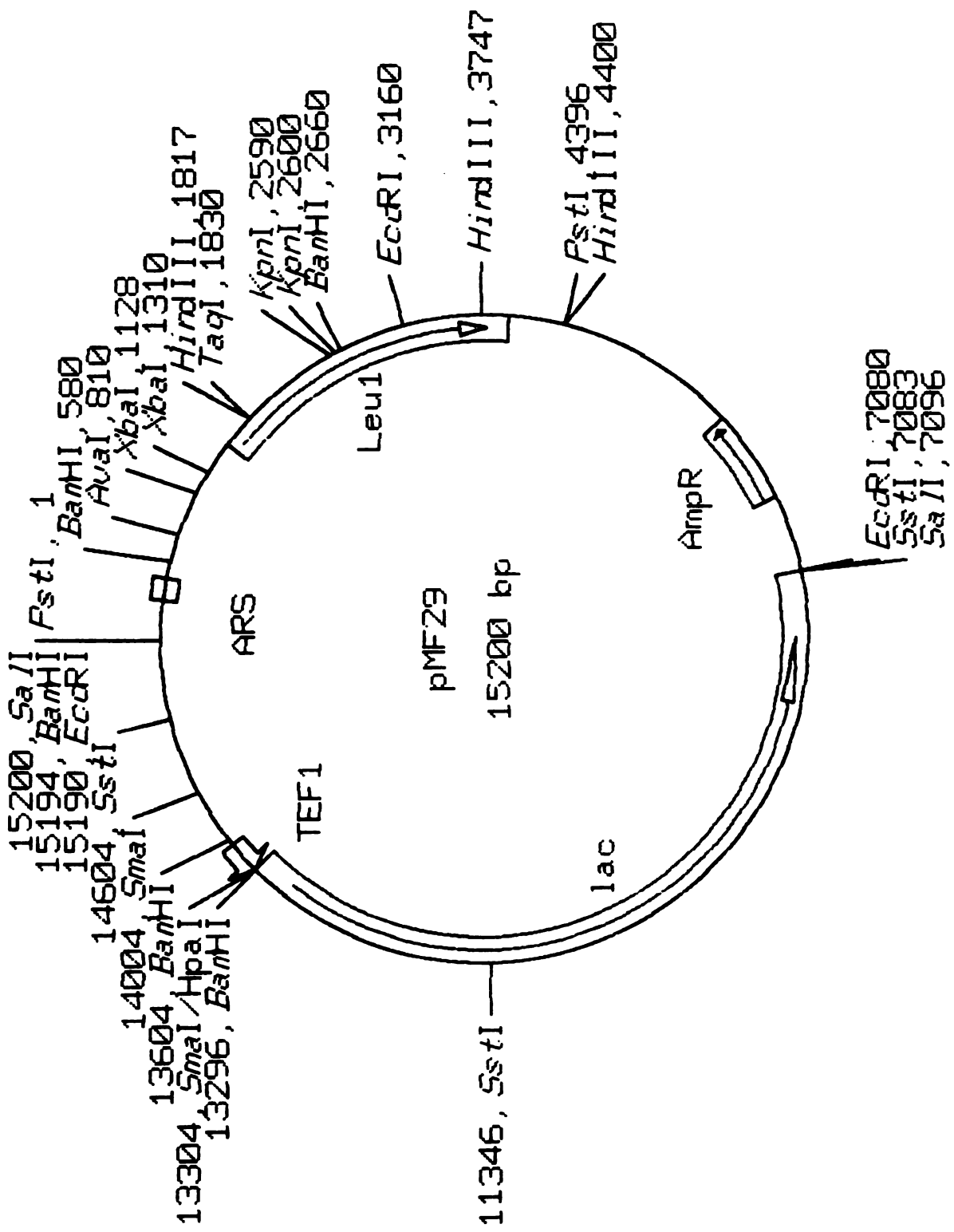

PstI, 1
BamHI, 580
AvaI, 810
XbaI, 1128
XbaI, 1310
HindIII, 1817
TaqI, 1830
KpnI, 2590
KpnI, 2600
BamHI, 2660
EcoRI, 3160
HindIII, 3747
PstI, 4396
HindIII, 4400
EcoRI, 7080
SstI, 7083
SalI, 7096
EcoRI, 7112
15200, SalI
ARS
Leu1
pMF25
15200 bp
AmpR
TEF1
lac
10970, SstI
9020, BamHI
9012, HpaI/SmaI
8712, BamHI
8312, SmaI
7712, SstI

Figure 7. Restriction map of plasmid pMF29.

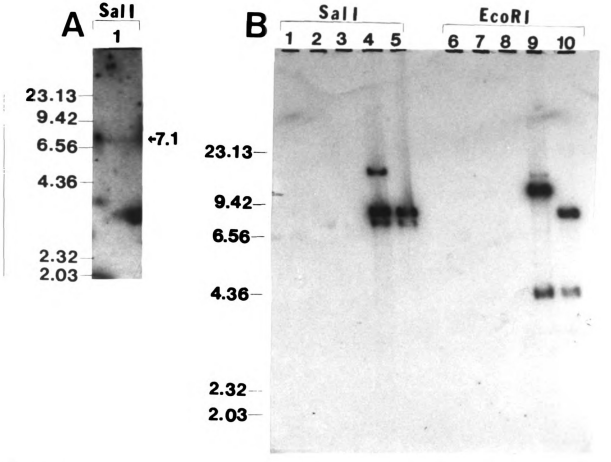

pMF29
15200 bp
ARS
TEF1
Leu1
lac
AmpR
1
BamHI, 580
AvaI, 810
XbaI, 1128
XbaI, 1310
HindIII, 1817
TaqI, 1830
KpnI, 2590
KpnI, 2600
BamHI, 2660
EcoRI, 3160
HindIII, 3747
PstI, 4396
HindIII, 4400
EcoRI, 7080
SstI, 7083
SalI, 7096
11346, SstI
13296, BamHI
13304, SmaI/HpaI
13604, BamHI
14004, SmaI
14604, SstI
15190, EcoRI
15194, BamHI
15200, SalI
PstI, 1

Plasmid pUC8 was also used as a probe in Southern analysis because it is nearly identical to the pUC13 backbone of the plasmid pLeu4 (containing the Leu1 gene), and has no homologous sequence in the host strain, Mucor racemosus ATCC 1216b R7B. Therefore, only DNA extracted from the transformants containing pLeu4, pMF25 or pMF29 was expected to hybridize with this probe. In DNA extracted from pMF25 transformants, the pUC8 DNA probe hybridized to a 7.1 Kb SalI restriction fragment and to a 3.9 Kb EcoRI restriction fragments. In DNA extracted from the pMF29 transformant, the pUC8 probe hybridized to a 7.1 Kb SalI restriction fragment and a 3.9 Kb EcoRI restriction fragments. No hybridization was detected in DNA extracted from the nontransformed host strain DNA or the pLeu4 transformant DNA (Figure 8, pp.65, 66). The explanation for this unexpected lack of hybridization to DNA extracted from pLeu4 transformants was because the pLeu4 transformant was grown under conditions without selective pressure which resulted in lower levels of plasmid DNA compared with DNA from pMF25 and pMF29 transformants which were grown under selective pressure. Since approximately equal amounts of DNA were loaded in all lanes, the plasmid pLeu4 is underrepresented and did not show up upon a short exposure of the autoradiography. In a previous experiment a 7.1 Kb SalI band was present after longer exposure of the Southern blot (Figure 8, pp.65, 66). Because these putative pMF25

Figure 8. Southern analysis of genomic DNA isolated from M. racemosus R7B, pLeu4 transformants, pMF25 and pMF29 transformants using a ^{32}P-labeled 6.2 Kb SalI fragment (lacZ) and pUC8 fragment as probes.

Panel A Lane 1: Genomic DNA (20 ug) of pLeu4 transformant was digested with SalI. The restriction fragments were separated by electrophoresis on a 0.8% agarose gel, transferred to a nitrocellulose filter and hybridized with a ^{32}P labeled pUC8 probe. The filter was washed at high stringency and exposed to X-ray film for 3 days at -70°C.

Panel B Genomic DNA (20 ug) was digested with SalI and EcoRI endonucleases. The restriction fragments were separated by electrophoresis on a 0.8% agarose gel, transferred to a nitrocellulose filter and hybridized with a ^{32}P-labeled 6.2 Kb SalI fragment containing the lacZ gene (from plasmid pMC1843) and pUC8 probe. The filter was washed at high stringency and exposed to X-ray film for 22 hours at room temperature. Lane 1 and 6: genomic DNA of M. racemosus R7B digested with SalI and EcoRI respectively. Lane 2, 3, 7 and 8: genomic DNA of two different pLeu4 transformants digested with SalI and EcoRI respectively. Lane 4 and 9: genomic DNA of pMF25 transformant digested with SalI and EcoRI respectively. Lane 5 and 10: genomic DNA of pMF29 transformant digested with SalI and EcoRI respectively.


A
Sal I
1
23.13
9.42
6.56
4.36
2.32
2.03
←7.1
B
Sal I
EcoR I
1 2 3 4 5 6 7 8 9 10
23.13
9.42
6.56
4.36
2.32
2.03

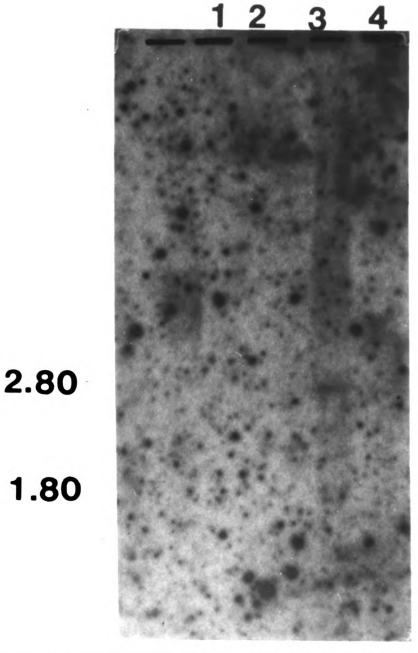
and pMF29 transformants could grow on YNB minimal medium combined with the result of Southern analysis suggested that the plasmids pMF25 and pMF29 were stable inside the host strain under selective growth conditions. Since the probes hybridized to the predicted restriction fragments, there were no obvious gene rearrangements after the plasmids were transformed and propagated in the Mucor host strain.

Northern Analysis of RNA Extracted from Mucor Transformants-pMF25, pMF29

Northern analysis was carried out to detect the level of transcription from the putative TEF1 promoter. RNA was extracted from the transformants. RNA samples (20 ug) were separated by electrophoresis on a 1% agarose-formaldehyde denaturing gel, transferred to a nitrocellulose filter, and hybridized to a 6.2 Kb lacZ gene probe (Figure 9, pp.68, 69). The probe hybridized to a heterogenous population of transcripts in RNA extracted from pMF25 transformant (lane 3) after 17 days of exposure but not to the other RNA samples. This hybridization data suggested that transcription of the lacZ gene occurred inside the Mucor host transformed with pMF25 but not pMF29. But, the level of transcript accumulation was so low that it was detectable only after a long exposure time (17 days). One possible explanation for this data is that there was no eukaryotic

Figure 9. Northern analysis of total RNA purified from M.racemosus R7B, a pLeu4 transformant, a pMF25 transformant and a pMF29 transformant using a ^{32}P labeled 6.2 Kb SalI fragment containing the lacZ gene as the probe (from pMC1843).

Total RNA (20 ug) was denatured with formamide/ formaldehyde, separated by electrophoresis on a 1% agarose formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P labeled 6.2 Kb SalI fragment (lacZ). The filter was washed at high stringency and exposed to X-ray film for 17 days. Lane 1: total RNA of M. racemosus R7B. Lane 2: total RNA of pLeu4 transformant. Lane 3: total RNA of pMF25 transformant. Lane 4: total RNA of pMF29 transformant.


1 2 3 4
2.80
1.80

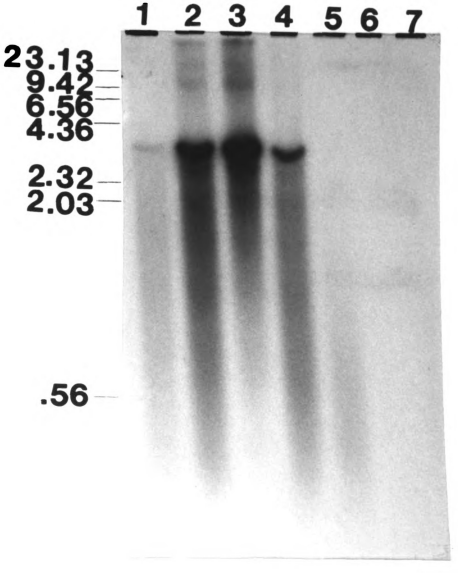
transcription termination signal included in plasmids pMF25 and pMF29 so transcription resulted in many sizes of mRNA. The lack of a processed 3' end of the transcript may also have contributed to the low level of transcript possibly because of mRNA instability. Because the data from Northern analysis were unclear, S1 Nuclease analysis and β-galactosidase assays were performed to detect transcription from the TEF1 promoter, and functional expression of a lacZ protein (β galactosidase).

S1-Nuclease Mapping of RNA Extracted from Transformants

Nuclease-S1 mapping was used to localize the start site of transcription of the TEF1/lacZ gene fusion. Total RNA was isolated from the Mucor transformants. The 3.2 Kb SacI fragment containing the TEF1 promoter region was gel purified from a restriction digest of plasmid pMF25 and used as the target DNA. Total RNA (100 ug) of each transformant (M.racemosus transformed with pLeu4, pMF25, pMF29) and the nontransformed host strain (M.racemosus ATCC 1216b R7B) was mixed with 0.1 ug of the 3.2 Kb template DNA. The nucleic acids were coprecipitated, heated to denature the DNA and allowed to hybridize and incubated with S1 nuclease. The DNA/RNA hybrids resistant to S1 digestion were resolved on a 1% agarose gel, transferred to a nitrocellulose filter and hybridized to the 6.2 Kb lacZ gene probe (Figure 10, pp.71).

Figure 10. S1-nuclease analysis of total RNA purified from M. racemosus R7B, a pLeu4 transformant, a pMF25 transformant, and a pMF29 transformant.

Total RNA (100 ug) was incubated with the 3.2 Kb SacI fragment containing the TEF1 promoter region (from pMF25) under conditions favoring RNA/DNA hybridization. The nucleic acids were coprecipitated, heated to denature the DNA, allowed to hybridize, and incubated with S1 nuclease. The DNA/RNA hybrids resistant to S1 digestion were resolved on a 1% agarose gel, transferred to a nitrocellulose filter and hybridized to a ^{32}P-labeled 6.2 Kb lacZ gene probe. Lane 1: total RNA from M. racemosus R7B plus the 3.2 Kb SacI DNA fragment. Lane 2: total RNA from pLeu4 transformant plus the 3.2 Kb SacI DNA fragment. Lane 3: total RNA from pMF25 transformant plus the 3.2 Kb SacI DNA fragment. Lane 4: total RNA from pMF29 transformant plus the 3.2 Kb SacI DNA fragment. Lane 5: tRNA plus the 3.2 Kb SacI DNA fragment. Lane 6: total RNA from pMF25 transformant. Lane 7: 3.2 Kb SacI DNA fragment.


1
2
3
4
5
6
7
23.13
9.42
6.56
4.36
2.32
2.03
.56

A similar band pattern was present in RNA from pMF25, pMF29 transformants (lanes 3, 4) and from a pLeu4 transformant (lane 2) which did not contain the TEF1 promoter region or lacZ gene. In addition, the size of this band (≈ 3.2 Kb) was similar to the size of the DNA template but not to the size of expected fragment (1.9 Kb) if the TEF1 promoter was functioning. These data suggested that the lacZ gene probe hybridized to target DNA reannealed to itself and not with the target DNA protected by the fusion transcript. These data also suggested that either the fusion transcript detected in pMF 25 transformants was subject to degradation in the Mucor cells or was produced in insufficient levels to detect by S-1 analysis.

β-Galactosidase Assay

Cell extracts of the pMF25, pMF29, and pLeu4 (control) transformants were analyzed to detect expression of β-galactosidase activity by using a β-galactosidase enzyme assay. The level of β-galactosidase activity detected in transformants was not consistently higher than control cells in which there appears to be a low level of endogenous β-galactosidase activity. Since we were unable to conclusively demonstrate function of the TEF1 promoter with the lacZ fusion, further experiments were conducted to investigate the function of the TEF1 promoter with a

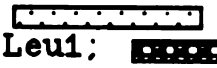
different reporter gene, the human growth hormone gene (hGH).

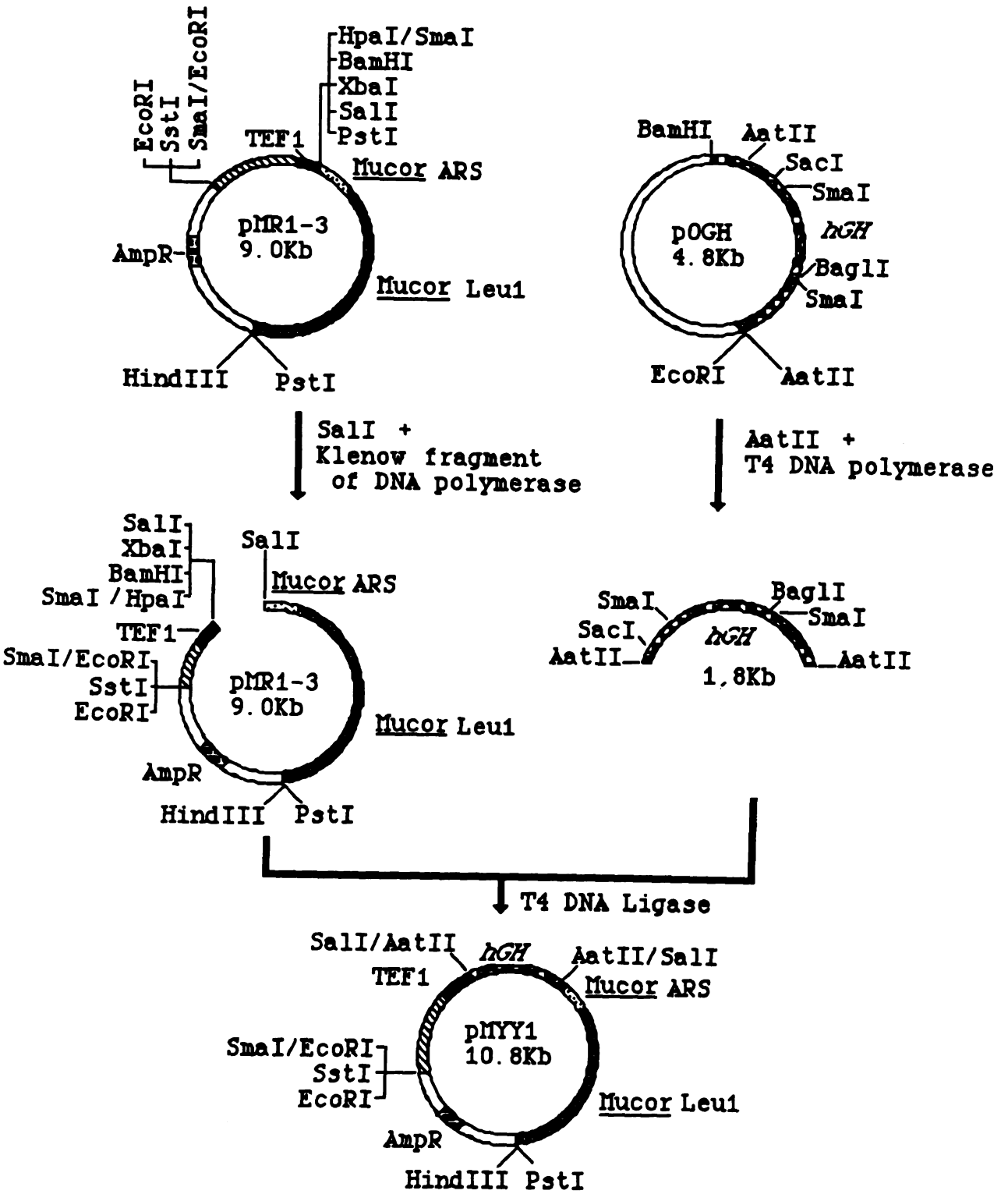
(2) TEF1/hGH Fusion Plasmids:

Construction of Gene Fusion Plasmids

A gene fusion was constructed between the Mucor promoter of TEF1, and the hGH (human growth hormone) gene in order to detect the level of transcription from the TEF1 promoter. The construction of this model expression vector is outlined in Figure 11 (pp.75, 76). The backbone for this construct was pMR1-3, a plasmid consisting of a 1.9 Kb EcoRI-HpaI restriction fragment containing the putative TEF1 promoter sequence plus the first 8 amino acids from the EF-1α protein, a pUC13 vector, and a Mucor leucine biosynthetic gene with a putative Mucor ARS (autonomous replication sequence). The plasmid pMR1-3 was cut with SalI endonuclease and the restriction sites were filled in with Klenow fragment of DNA polymeraseI to produce blunt ends. A 1.8 Kb AatII fragment containing the hGH gene was gel purified from a restriction digest of plasmid p0GH and the restriction sites of this fragment were treated with T4 DNA polymerase to generate blunt ends. This blunt-ended AatII fragment was ligated into the blunt ended SalI site of pMR1-

Figure 11. Construction of plasmid pMYY1.

The actual manipulations required for this construction are described in detail in Results. The plasmids pMR1-3 and pOGH are described in Materials and Methods. The regions representing particular DNA fragments are labeled:  , TEF1 promoter region;  , ampicillin resistance gene;  , Mucor ARS;  , Mucor Leu1;  , hGH (human growth hormone) gene.


HpaI/SmaI
BamHI
XbaI
SalI
PstI
EcoRI
SstI
SmaI/EcoRI
TEF1
Mucor ARS
pMR1-3
9.0Kb
AmpR
Mucor Leu1
HindIII
PstI
BamHI
AatII
SacI
SmaI
hGH
pOGH
4.8Kb
BglII
SmaI
EcoRI
AatII
SalI +
Klenow fragment
of DNA polymerase
AatII +
T4 DNA polymerase
SalI
XbaI
BamHI
SmaI /HpaI
SalI
Mucor ARS
TEF1
SmaI/EcoRI
SstI
EcoRI
pMR1-3
9.0Kb
Mucor Leu1
AmpR
HindIII
PstI
SmaI
SacI
AatII
BglII
SmaI
hGH
AatII
1.8Kb
T4 DNA Ligase
SalI/AatII
hGH
AatII/SalI
TEF1
Mucor ARS
pMYY1
10.8Kb
SmaI/EcoRI
SstI
EcoRI
Mucor Leu1
AmpR
HindIII
PstI

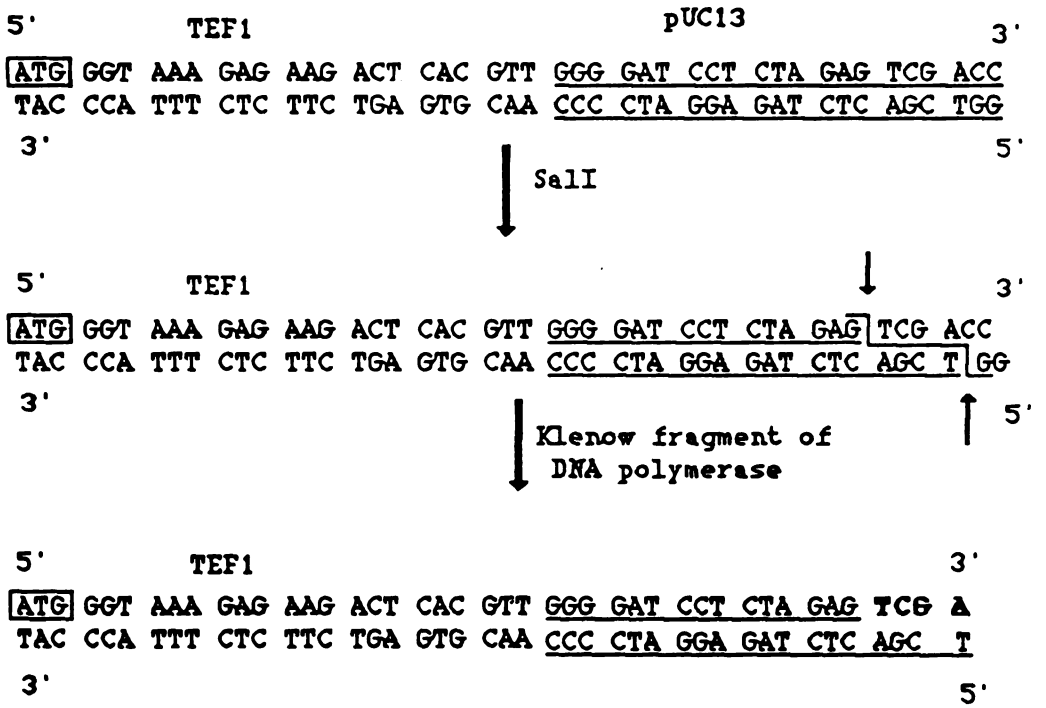
3 adjacent to the TEF1 promoter. This fusion generated a continuous open reading frame between the ATG initiation codon plus the first 8 amino acids of TEF1 gene and the hGH gene beginning with amino acid number seven of the signal peptide of the pre-hGH protein. The transcription termination signal was therefore provided by the hGH gene while the transcription initiation signal was provided by the TEF1 gene (Fig. 12, pp.78, 79, 80).

Isolation and Analysis of Constructed Plasmids

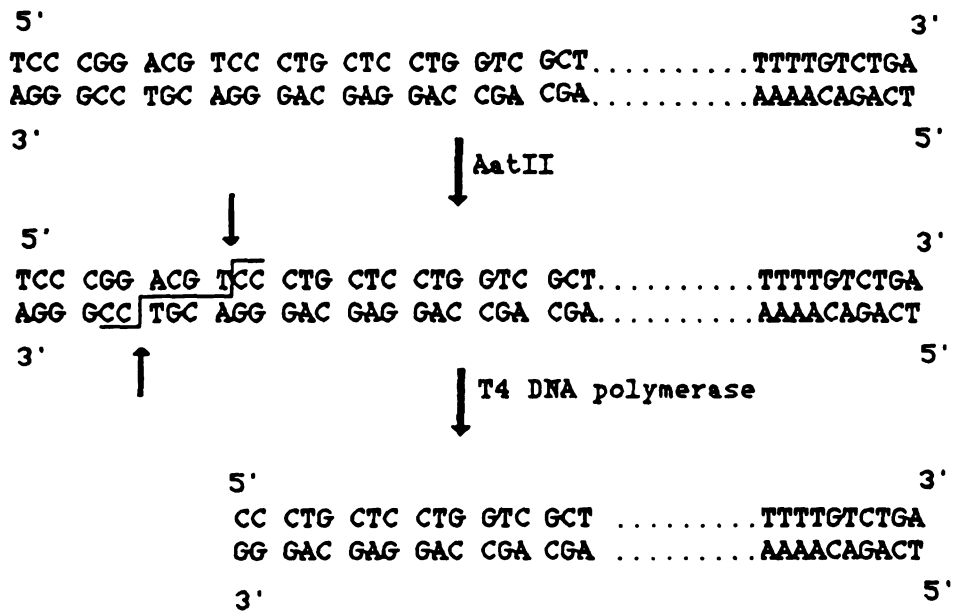
Newly constructed plasmids were transformed into E. coli DH 5α to propagate the plasmid molecules. Transformants, thought to contain the desired recombinant plasmid, grew on LB plates supplemented with ampicillin (50 ug/ml) and were further identified by the colony hybridization procedure with a ^{32}P-labeled 1.8 Kb AatII hGH gene probe (Fig. 13, pp.81). Fifteen of 330 colonies were found to contain hGH gene inserts (≈ 5% ligation efficiency). After single colony isolation, plasmid DNA containing the hGH gene was obtained from E. coli clones using the "miniprep" method. Samples of the resulting plasmid DNAs were digested with EcoRI and SacI restriction endonucleases and analyzed by electrophoresis through a 1% agarose gel. SacI endonuclease was expected to distinguish between the two orientations of the insert by the generation

Figure 12. Construction of plasmid pMYY1. (nucleotide sequence)

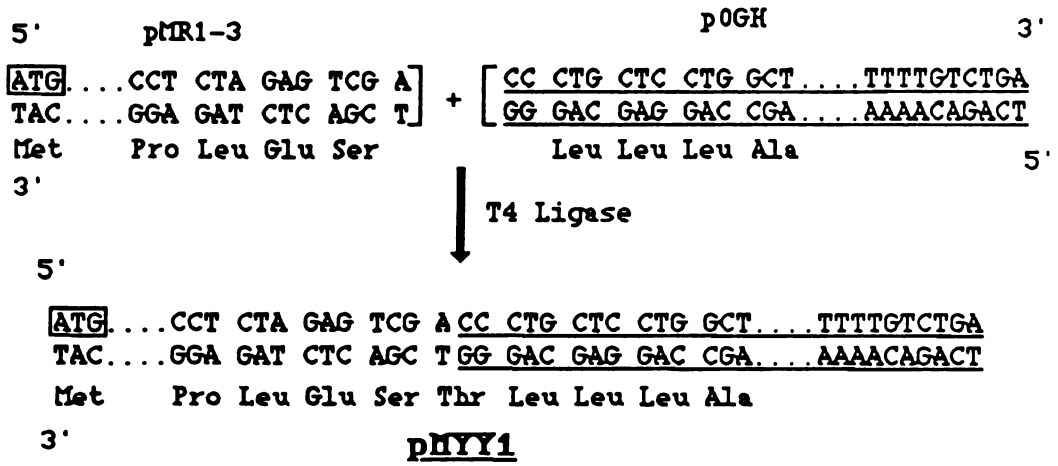
pMR1-3


5'
TEF1
pUC13
3'
ATG GGT AAA GAG AAG ACT CAC GTT GGG GAT CCT CTA GAG TCG ACC
TAC CCA TTT CTC TTC TGA GTG CAA CCC CTA GGA GAT CTC AGC TGG
3'
5'
SalI
5'
TEF1
3'
ATG GGT AAA GAG AAG ACT CAC GTT GGG GAT CCT CTA GAG TCG ACC
TAC CCA TTT CTC TTC TGA GTG CAA CCC CTA GGA GAT CTC AGC T GG
3'
5'
Klenow fragment of
DNA polymerase
5'
TEF1
3'
ATG GGT AAA GAG AAG ACT CAC GTT GGG GAT CCT CTA GAG TCG A
TAC CCA TTT CTC TTC TGA GTG CAA CCC CTA GGA GAT CTC AGC T
3'
5'

pOGH


5'
3'
TCC CGG ACG TCC CTG CTC CTG GTC GCT.....TTTTGTCTGA
AGG GCC TGC AGG GAC GAG GAC CGA CGA.....AAAACAGACT
3'
5'
AatII
5'
3'
TCC CGG ACG TCC CTG CTC CTG GTC GCT.....TTTTGTCTGA
AGG GCC TGC AGG GAC GAG GAC CGA CGA.....AAAACAGACT
3'
5'
T4 DNA polymerase
5'
3'
CC CTG CTC CTG GTC GCT .....TTTTGTCTGA
GG GAC GAG GAC CGA CGA .....AAAACAGACT
3'
5'

pMYY1


5'
pMR1-3
pOGH
3'
ATG....CCT CTA GAG TCG A
TAC....GGA GAT CTC AGC T
+
CC CTG CTC CTG GCT....TTTTGTCTGA
GG GAC GAG GAC CGA....AAAACAGACT
Met Pro Leu Glu Ser
Leu Leu Leu Ala
5'
3'
T4 Ligase
5'
ATG....CCT CTA GAG TCG A CC CTG CTC CTG GCT....TTTTGTCTGA
TAC....GGA GAT CTC AGC T GG GAC GAG GAC CGA....AAAACAGACT
Met Pro Leu Glu Ser Thr Leu Leu Leu Ala
3'

pMYY1

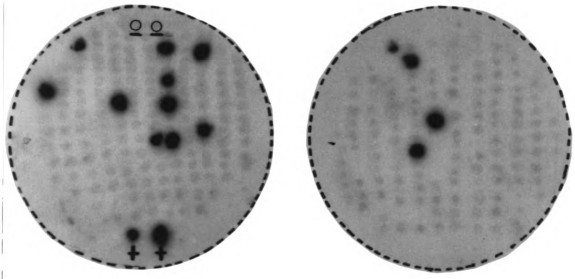


Figure 13. Colony hybridization with an hGH gene fragment as probe.

E. coli cells were grown on a nitrocellulose filter, lysed, denatured, neutralized and hybridized with the hGH gene probe to identify the cells which contained hGH gene inserts. Filters were washed under high stringency conditions and exposed to X-ray film for 2.5 hours at room temperature. "+" represents the colony containing the hGH gene insert as a positive control. "-" represents the colony containing plasmid pLeu4 as a negative control.

of three DNA fragments (8.7 Kb, 1.5 Kb, 0.6 Kb) in one orientation (in frame fusion) and three unique DNA fragments (7.3 Kb, 2.9 Kb, 0.6 Kb) in the other orientation. Restriction endonuclease analysis showed that two out of ten clones contained the correct orientation of the insert, 0 out of ten clones contained the insert in the opposite orientation and eight out of ten clones contained multiple inserts in several combinations. The two clones thought to contain the correct fusion construct were selected to perform detailed restriction analysis with different endonucleases including EcoRI, SacI, SmaI, SmaI & SacI, BamHI and HindIII (Fig. 14, pp.83, 84) to confirm the proper plasmid construction. The predicted restriction map (Fig. 15, pp.85, 86) of this constructed plasmid (named pMYY1) was confirmed according to the data of the restriction analysis.

Plasmid pMYY1 was amplified using the large-scale protocol and isolated by cesium chloride density gradient centrifugation. The purified plasmid DNA was used to transform protoplasts of Mucor racemosus R7B.

Figure 14. Restriction analysis of plasmid pMYY1

Plasmid DNA of pMYY1 was digested with different restriction endonucleases and the restriction fragments were separated by electrophoresis on a 1% agarose gels. Lane 1: undigested plasmid pMYY1. Lane 2, 3, 4, 5, 6, 7: plasmid DNA of pMYY1 digested with EcoRI, SacI, SmaI, SacI and SmaI, BamHI and HindIII endonucleases respectively.

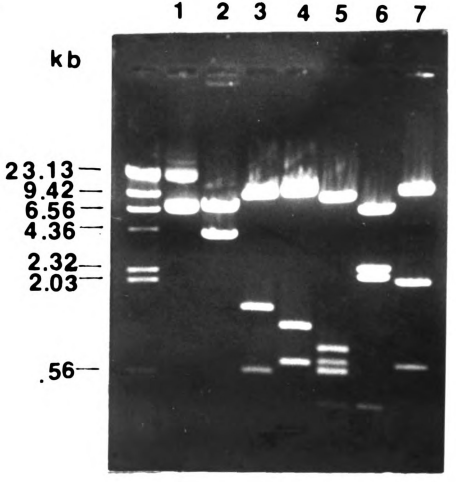
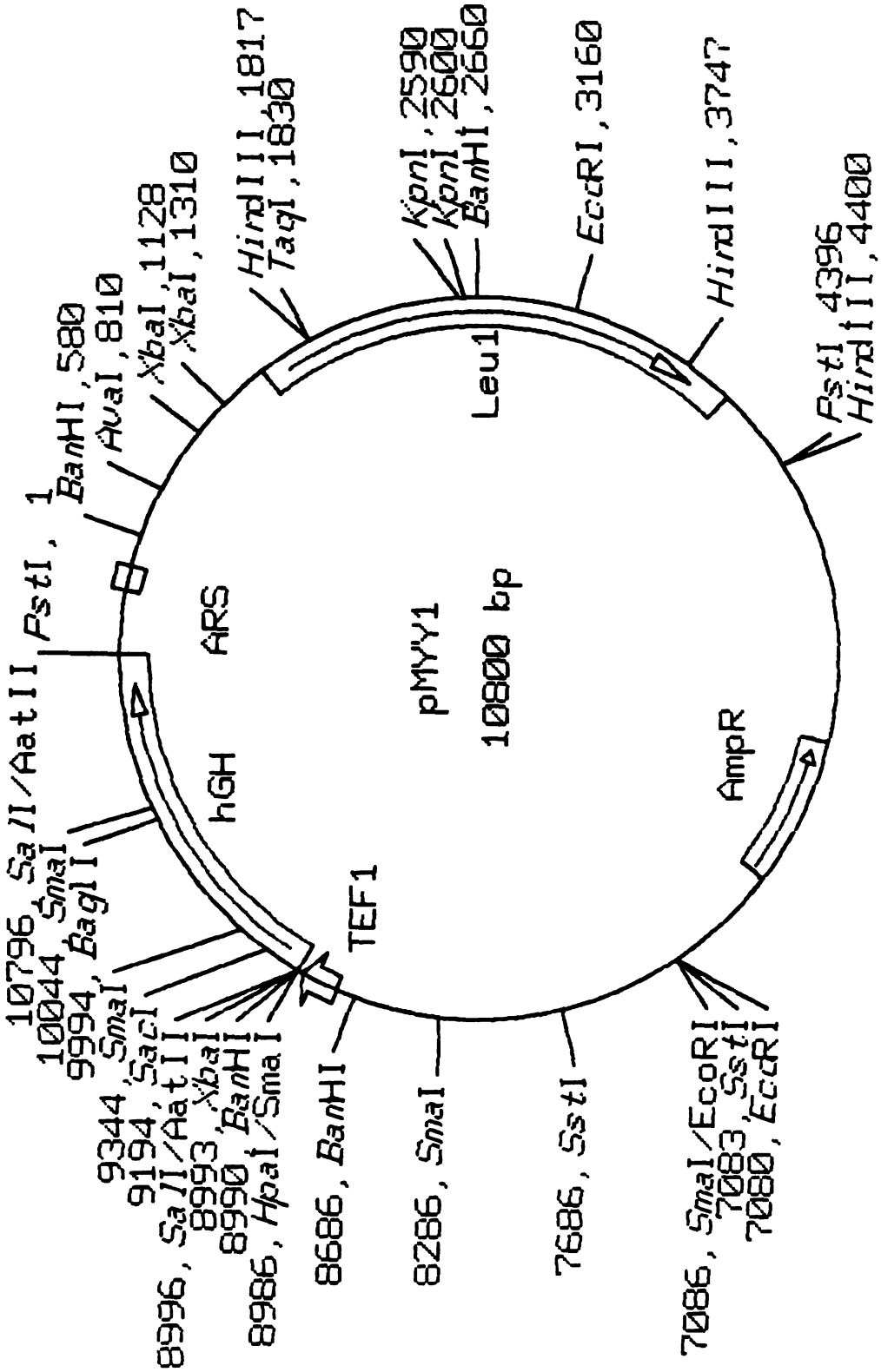

1 2 3 4 5 6 7
kb
23.13
9.42
6.56
4.36
2.32
2.03
.56

Figure 15. Restriction map of plasmid pMYY1.


pMYY1
10800 bp
hGH
ARS
Leu1
AmpR
TEF1
1, BamHI
580, AvaI
810, XbaI
1128, XbaI
1310, HindIII
1817, TaqI
1830
2590, KpnI
2600, KpnI
2660, BamHI
3160, EcoRI
3747, HindIII
4396, PstI
4400, HindIII
7080, EcoRI
7083, SstI
7086, SmaI/EcoRI
7686, SstI
8286, SmaI
8686, BamHI
8986, HpaI/SmaI
8990, BamHI
8993, XbaI
9194, SalI/AatII
9344, SacI
9944, SmaI
9994, BglII
10044, SmaI
10796, SalI/AatII
PstI

Transformation of Mucor racemosus R7B with pMYY1 and pMR1-3

M. racemosus ATCC 1216b R7B was transformed with the model expression plasmid pMYY1 to demonstrate the function of the TEF1 promoter by measuring the expression of the hGH gene in Mucor cells. Approximately 50% of the sporangiospores of M. racemosus R7B inoculated in YPG broth germinated after 5.5 hours of incubation (3-5 spore diameters). Protoplasts were generated (about 40% of germlings) after 1 hour of cell wall digestion with Chitosanase and Novozyme 234. The protoplasts were transformed with the plasmid pMYY1. Eight putative transformants were obtained after 2 - 3 days growth on YNB (pH 3.0) minimal medium (without leucine). The protoplasts of M. racemosus R7B were also transformed with pMR1-3 containing no hGH gene as the control. Two putative transformants were obtained after 2-3 days growth on YNB (pH 3.0) minimal plates. The frequency of protoplast regeneration of M. racemosus ATCC 1216b R7B was 40%. The frequency of transformation of pMYY1 was 10 colonies/ug DNA/10^6 protoplasts, and the frequency of transformation of pMR1-3 was 2.5 colonies/ug DNA/10^6 protoplasts. After single colony isolation, spore stocks were produced from four of the putative pMYY1 transformants and two of the putative pMR1-3 transformants.

Southern Analysis of DNA Extracted from Mucor Transformants

Genomic DNA of four putative pMYY1 transformants and one pMR1-3 transformant (control) was extracted and digested completely with different restriction endonucleases (10 ug, per lane). DNA restriction fragments were separated by electrophoresis through a 0.8% agarose gel and transferred to a nitrocellulose filter. The 1.8 Kb AatII fragment containing the hGH gene was radioactively labeled with ^{32}P and used to hybridize to the nitrocellulose filter for 23 hours. The filter was washed under high stringency conditions and exposed to X-ray film (Fig. 16, pp.90, 91). The 1.8 Kb AatII hGH gene probe hybridized to a 2.4 Kb BamHI restriction fragment and to an 8.2 Kb HindIII fragment in DNA from all 4 putative pMYY1 transformants. In the control transformant (pMR1-3), one very high molecular weight band was present on the X-ray film after a 23 hr exposure. One possible explanation for this unpredicted band is the non-specific binding of the probe to catenated pMR1-3 plasmids. Although the result of the control transformant was not as we expected, the data from Southern analysis confirmed the entry of pMYY1 into the Mucor host strain during transformation.

The same nitrocellulose filter was reprobed with a 1.9 Kb HindIII fragment from the pMR1-3 vector containing the

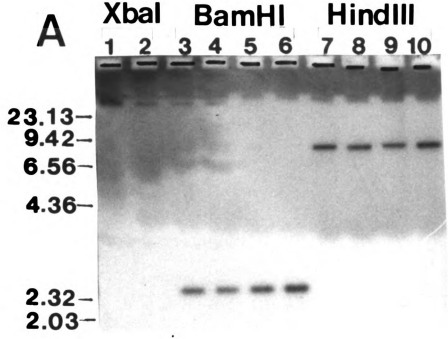
Leu1 gene (without removing the original hGH probe). The Leu1 gene probe hybridized to two BamHI fragments (2.1 Kb, 6.0Kb), to one HindIII fragment (1.9 Kb) in DNA isolated from pMYY1 transformants and to one XbaI fragment (7.7 Kb) in DNA isolated from the pMR1-3 transformant (Fig. 16, pp.90, 91). The ability of these putative transformants to grow on YNB minimal medium combined with the Southern analysis data demonstrated that the plasmids pMYY1 and pMR1-3 were present and stable inside the host strain when grown under selective pressure. Because the hybridization patterns observed in Southern analysis were identical to the restriction pattern of pure plasmid DNA, no obvious gene rearrangements occurred during transformation or propagation in the Mucor host strain.

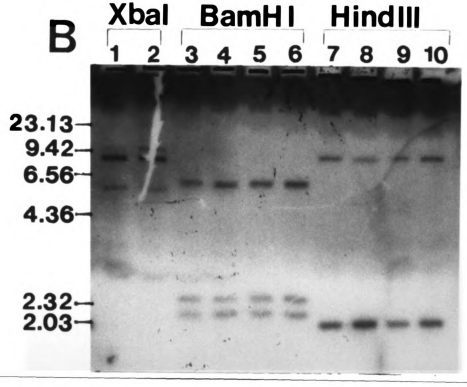
Northern Analysis of RNA Extracted from Mucor Transformants

Northern analysis was performed to detect the accumulated hGH transcript level to demonstrate the function of the putative TEF1 promoter. Total RNA was prepared from cells transformed with pMYY1 and pMR1-3. RNA samples (20 ug) were separated by electrophoresis on a 1% agarose-formaldehyde gel, and transferred to a nitrocellulose filter. The 1.8 Kb AatII hGH gene fragment was radioactively labeled with ^{32}P and used to hybridize to this Northern blot. After two non-specific washes and one high

Figure 16. Southern analysis of genomic DNA isolated from four pMYY1 transformants and pMR1-3 transformant (control) using a ^{32}P labeled 1.8 Kb AatII fragment (from p0GH) and 1.9 Kb HindIII fragment (from pMR1-3) as probes.

Genomic DNA (10 ug) was digested with different restriction endonucleases. The restriction fragments were resolved by electrophoresis on a 0.8% agarose gel, transferred to a nitrocellulose filter and hybridized with a ^{32}P labeled 1.8 Kb AatII fragment containing hGH gene (for Panel A), and a 1.9 Kb HindIII fragment containing the Leu1 gene (without removing original hGH probe; Panel B). The filter was washed at high stringency and exposed to X-ray film for 23 hours (Panel A) and 48 hours (Panel B) at room temperature. Lane 1, 2 of Panel A and Panel B: genomic DNA of pMR1-3 transformant digested with XbaI endonuclease. Lane 3, 4, 5, 6 of Panel A and Panel B: genomic DNA of four pMYY1 transformants digested with BamHI endonuclease. Lane 7,8,9,10 of Panel A and Panel B: genomic DNA of four pMYY1 transformants digested with HindIII endonuclease.


A
XbaI
BamHI
HindIII
1 2 3 4 5 6 7 8 9 10
23.13
9.42
6.56
4.36
2.32
2.03


B
XbaI
BamHI
HindIII
1 2 3 4 5 6 7 8 9 10
23.13
9.42
6.56
4.36
2.32
2.03

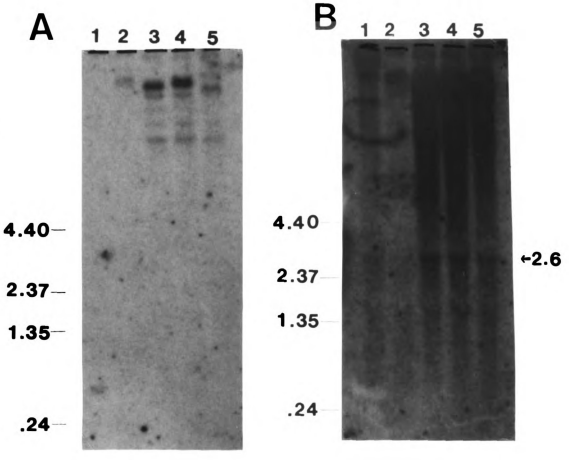
stringency final wash, the filter was analyzed by autoradiography (Fig. 17, pp.94, 95). After 9 days of exposure, several high molecular weight hybridization bands (30 - 100 Kb) were present on the lanes loaded with RNA extracted from the pMYY1 transformants but not on the lane loaded with RNA from the pMR1-3 transformant. These large hybridization bands did not correspond to the predicted size (≈ 1.0-1.2 Kb) for the mRNA derived from the hGH gene. One possible explanation for these hybridization data was the lack of function of the hGH gene transcription termination signal inside Mucor cells which resulted in production of high molecular weight, heterogeneous transcripts. Another possible explanation was that the RNA samples extracted from pMYY1 transformants were contaminated with plasmid DNA (pMYY1) and resulted in hybridization between concatamers of the plasmid DNA and the hGH gene probe. In order to distinguish between these possibilities and to show that the RNA samples were not degraded, a Leu1 gene probe was used to probe the same Northern blot. A previous study showed that the size for the mature mRNA derived from the Leu1 gene was about 2.5 Kb (Wang, S.Y., unpublished) which agrees closely with the size determined by Roncero et al., (unpublished). If only one hybridization band (≈ 2.5 Kb) appeared in the lane with pMR1-3 transformant RNA in Northern analysis (using Leu1 gene as the probe) it would mean that the high molecular weight bands observed previously were the result

of hybridization of transcripts of the hGH gene to the hGH gene probe. If hybridization to high molecular weight nucleic acid was also present in the lane with the RNA sample prepared from pMR1-3 transformant, it would suggest that the previous data was due to plasmid DNA contamination of the RNA sample.

The data from this experiment are seen in Figure 17 (pp .94, 95). Several high molecular weight bands were observed in the lane containing the RNA sample from the pMR1-3 transformant. In addition, the probe hybridized to a single transcript of approximately 2,600-nucleotides in lane 1 (containing the RNA sample from the pMR1-3 transformant) and lanes 3,4, 5 (RNA sample extracted from the pMYY1 transformants). The size of this transcript corresponded to the expected size for the mature mRNA derived from the Leu1 gene. One possible reason for the absence of the 2.6 Kb transcript in lane 2 which contained RNA from one pMYY1 transformant was because of low quantity of total RNA loaded in lane 2 (10 ug; compared with 20 ug in all other lanes). These data indicate that the large molecular weight hybridization signals resulted from contamination of the RNA samples with plasmid DNA and that the RNA samples were not degraded during the purification procedure. The data presented in Southern and Northern analyses suggested that the TEF1 promoter sequence subcloned from the TEF1 gene was

Figure 17. Northern analysis of total RNA isolated from four pMYY1 transformants and a pMR1-3 transformant using a ^{32}P labeled 1.8 Kb AatII fragment containing the hGH gene (from pOGH) and a 1.9 Kb HindIII fragment containing the Leu1 gene (from pMR1-3) as probes.

Total RNA (10-20 ug) was denatured with formamide/formaldehyde, separated by electrophoresis on a 1% agarose-formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with a ^{32}P labeled 1.8 Kb AatII fragment (for Panel A), and 1.9 Kb HindIII fragment (without removing original hGH probe; Panel B). The filter was washed at high stringency and exposed to X-ray film for 9 days (Panel A) and 16 days (Panel B). Lane 1 of Panel A and Panel B: total RNA of pMR1-3 transformant. Lane 2, 3, 4, 5 of Panel A and Panel B: total RNA of four pMYY1 transformants.


A
1 2 3 4 5
4.40
2.37
1.35
.24
B
1 2 3 4 5
4.40
2.37
1.35
.24
←2.6

not efficient to drive the high level expression of a heterologous gene.

DISCUSSION

Genetic transformation of Mucor racemosus was achieved by incubating protoplasts generated from a leucine requiring strain with recombinant plasmid DNA (pMYY1, pMR1-3, pMF25 or pMF29) in the presence of polyethylene glycol and $CaCl_2$. The transformation frequency of M.racemosus with pMYY-1 was 10 transformants / ug DNA / 10^6 protoplasts which was a much lower frequency than that obtained for pMCL1302 by Van Heeswijck (1984; 600 transformants / ug DNA / 3.2 x 10^6 protoplasts). This result might due to the fact that the mixtures containing protoplasts and plasmid pMYY1 were plated at too high of a cell density or because glucose was inadvertently left out of the growth medium resulting in a decreased cell viability.

Because the spontaneous reversion frequency of R7B to Leu^+ is< $1.7x10^{-8}$ (Van Heeswijck, 1984) and the number of protoplasts used in transformation was so low (< 2 x 10^6), the colonies harvested from the YNB medium after transformation were thought to be transformants. This hypothesis was confirmed by Southern analysis which showed that the plasmids pMYY1, pMR1-3, pMF25 or pMF29 were present in the fungal cells after transformation. The data also suggested that there was no obvious genetic rearrangement of the molecules after the plasmids were transferred into host

cells. There are three possible fates of a plasmid which enters a host cell. A plasmid may replicate autonomously. It may integrate into chromosomal DNA randomly by nonhomologous recombination or it may integrate into chromosomal DNA at specified sites via homologous recombination. The results of our Southern analysis suggested that the plasmids pMYY1, pMR1-3, pMF25 and pMF29 did not integrate into genome of the host cells, but existed as free plasmid molecules. These data agreed with similar results observed by Van Heeswijck (1986); i.e. transformed DNA plasmids are present as discrete extrachromosomal DNA in Mucor racemosus. One experiment which will be useful to confirm our conclusion will be to isolate genomic DNA of Mucor transformants (pMF25, pMR1-3, pMF29 or pMYY-1) and utilize this directly to transform E. coli DH 5α. If the plasmid can be rescued from E. coli cell in its original form, it would support the notion that plasmid pMF25, pMF29, pMR1-3, or pMYY1 exist as self-replicating molecules inside the Mucor cells.

Other experimental data have demonstrated that pLeu4 transformants tend to lose their plasmids at a fast rate when they are grown without selective pressure (YPG rich medium containing leucine) (Juili Lin, M.S. Thesis). This data also agrees with results reported by Van Heeswijck (1986). Only 5% of viable sporangiospores isolated from

pMCL1302 transformants were Leu+ after the first transfer to complete medium, compared with 45% viability when growth of spores was continued under selective conditions (YNB medium). One possible reason for this observation could be that the plasmids do not integrate into genome of the host cell, so they would not be segregated equally to daughter cells during mitosis. Because gene stability is essential to an effective heterologous protein expression system, more data are required to determine the fate of transforming plasmid DNA inside M.racemosus host cells.

It would be advantageous from a stability standpoint if a certain percentage of vector molecules do integrate into the chromosome. The following experiment is currently being conducted to test for this possibility. Transformant cells were grown without selective pressure for several generations followed by replica plating of colonies onto selective minimal medium to generate isolated colonies. This procedure was repeated several times. If any plasmid DNA integrated into the genome of the host, it should be maintained stably and detected after inoculation of spores back onto YNB minimal medium. If the plasmids did not integrate into the genome of host, the high level of segregational instability in Mucor (Van Heeswijck, 1986) for cells grown in the absence of selective pressure would cause the cells to rapidly lose their plasmids resulting in the

inability to recover cells with the Leu+ phenotype upon plating spores onto YNB.

The results of these experiments showed that one clone derived from the pMF25 transformant was stable after several transfers to YNB medium. One possible explanation for these data is that plasmid molecules integrated into the chromosomal DNA. Another explanation is that a wild type M. racemosus 1216b contaminant was isolated which resulted in extremely stable Leu+ phenotype. On the other hand, the clone derived from the pMF29 transformant was unable to grow on the YNB minimal plate even before passage on YPG medium (Linz, J., unpublished). The loss of the plasmid pMF29 during manipulations in generating a spore stock may explain the data of Northern analysis in which transcription of the TEF1/lacZ gene occurred inside the Mucor host transformed with pMF25 but not in the pMF29 transformant. We must now perform Southern analysis on these clones to determine the location of plasmid DNA.

The Mucor TEF1 promoter was chosen in our model system because this gene is a constitutive, highly expressed gene encoding elongation factor 1α which is one of the most abundant proteins present in M. racemosus. Because of the high level transcription of this gene, the TEF1 promoter was suspected to be a very strong promoter. In order to

determine the function of this promoter, lacZ and hGH reporter genes were fused to TEF1 promoter. The constructed plasmids were pMF25, pMF29 and pMYY1. The results of Southern analysis confirmed the presence of plasmids pMF25, pMF29 or pMYY1 in transformants. The data of Northern analysis suggested that a low level of fusion transcript was produced inside the pMF25 transformant but not in pMF29 or pMYY1 transformants. These data lead us to conclude that the TEF1 promoter sequence subcloned from the TEF1 gene was not efficient to drive the high level expression of the heterologous genes tested in this study.

Some promoters seem to be recognized by RNA polymerase in a relatively general mechanism while other promoters are not adequate to support transcription by themselves. Specific ancillary factors such as enhancers are needed for initiation to occur efficiently (Lewin, 1983). We propose here that the TEF1 promoter sequence utilized in this study lacks an important recognition site required for specific transcription factors required for initiation of transcription. A previous study (Linz, et al., 1986) determined that EF-1α in Mucor racemosus is encoded by three genes (TEF1, TEF2, TEF3) located at unique positions in the genome. Nucleotide sequence analysis of the genes indicated that these three genes share a high degree of similarity not only in the coding portion of the gene, but also in the 3'

intron. All three genes have a conserved intron near the 3' end of the coding region. In addition, TEF2 and TEF3, but not TEF1, each have an intron located near the 5' end of the coding region. Conservation of the nucleotide sequence within the 3' intron suggests that it might play an important role for facilitating transcription from the TEF1 promoter.

To investigate this hypothesis, the following experiments will be conducted. A portion of the hGH gene open reading frame (≈ 400-500 bp) will be ligated in frame into the intact TEF1 gene in the coding region downstream from the 3' intron. This construct will be ligated into the pLeu4 plasmid and used to transform M.racemosus R7B. Southern and Northern analyses will be carried out on nucleic acid purified from the transformants. If stable transcripts of this gene fusion are detected in Northern analysis using an hGH gene probe, it would suggest that specific nucleotides sequences between the ATG initiation codon and transcription termination signal are needed for the initiation of transcription from the TEF1 promoter. Deletion of nucleotides sequences from the TEF1 gene could be accomplished using several convenient restriction sites from upstream and downstream of the hGH gene insert to determine the position of important ancillary factors. If the intron is important in facilitating the initiation of

transcription and its position is not strictly limited, the nucleotide sequence which contains the promoter region (275 bp nucleotides sequence upstream from ATG initiation codon) and the intron will be used as a promoter for the further experiments. Studies such as heterologous protein expression/secretion in Mucor will be performed. If the presence of exonI or exonII of the TEF1 gene is required for the function of TEF1 promoter or if the distance between the promoter and the intron is fixed, the TEF1 promoter will not be convenient to be a promoter in our model system. Another promoter (for example, Leu1 promoter) will be considered to perform further experiments.

If no transcripts of this gene fusion are detected in Northern analysis using the hGH gene, it would suggest that transcription of the TEF1 gene involves sequences at a distance from the gene itself. For example, nucleotide sequences a large distance upstream from the ATG initiation codon may be required in this construct. Experiments such as inclusion of additional nucleotides from the upstream region of the TEF1 promoter sequence could be conducted. Another possible reason that the TEF1 promoter may not function is that the autonomously replicating vector lacks genomic context (ie chromatin, nucleosomes structure, etc.) required for TEF1 gene expression.

SUMMARY

Since the experimental results from the original TEF1/lacZ fusion transformants did not conclusively demonstrate function of the Mucor TEF1 promoter, this research project was designed to investigate the function of the TEF1 promoter with the human growth hormone gene (hGH) as a reporter gene. The hGH gene was inserted into the vector pMR1-3 at a position adjacent to the TEF1 promoter. The correct construction of the resulting plasmid pMYY1 was confirmed by restriction endonuclease analysis. The plasmid was then used to transform M. racemosus R7B. Plasmid pMYY1 was observed to be present in transformants with no obvious DNA rearrangements. However, there was no detectable transcript of the hGH gene produced in Mucor cells. The results of this study suggested that TEF1 promoter was not efficient to drive the high level expression of a heterologous gene. Further studies will focus on finding ancillary factors necessary for the function of the TEF1 promoter and to determine if this promoter is suitable in our model system.

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