To the Graduate Council:

I am submitting herewith a dissertation written by Clarence C. Morse entitled "Isolation and Partial Characterization of the ARO1 Gene of Saccharomyces cerevisiae." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Sankar Mitra

Sankar Mitra, Major Professor

We have read this dissertation
and recommend its acceptance:

Frank W. Larimer

Frank Gaertner

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C. W. Minkel

The Graduate School

ISOLATION AND PARTIAL CHARACTERIZATION OF THE *ARO1* GENE OF *SACCHAROMYCES CEREVISIAE*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Clarence C. Morse

August 1983

DEDICATION

To my wife Cindy, Sasha von Pluto Amina,

Kassim and Schroeder

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I extend my deepest and most sincere thanks to those friends and researchers who have repeatedly given of themselves in an effort that ultimately led to the focus of my many years of education--a chance to do biological research as a professional, and the opportunity to examine a few of the many unknowns; and perhaps, thereby contribute a small piece of the "living puzzle." I express additional thanks to Drs. Tex Barnett and Ed Hiss for their continued "support," advice, and friendship over the past years--without which, the pursuit of my Ph. D. at The University of Tennessee Oak Ridge Graduate School of Biomedical Sciences would not have been possible. Special thanks and appreciation I extend to the man who contributed most significantly and influentially to my development as a scientist, my good friend, and major professor, Dr. Sankar Mitra. To Drs. Frank Larimer, Frank Gaertner, Abe Hsie, and Larry Boone, I extend my undying gratitude for the use of their laboratory facilities, for their constructive criticisms, and for their time and efforts which each of these people contributed to my training in the area of recombinant DNA technology, and to my emergence as a competitive researcher in molecular biology. To my other committee members, Drs. Robert Fujimura, Salil Niyoqi and Lee Shugart, I extend my thanks for their constructive support and advice throughout my graduate research, and all during the preparation of the final version of this dissertation. Special thanks to Dr. Chin Yi Ou for his

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ABSTRACT

The ARO1 cluster-gene of Saccharomyces cerevisiae is thought to be a single transcriptional unit encoding one polypeptide and five sequential enzymatic activities involved in the biosynthesis of the aromatic amino acids. The ARO1 cluster-gene was previously isolated by complementation in Saccharomyces cerevisiae following transformation with a comprehensive yeast DNA library of BamHI restriction fragments inserted into the shuttle vector YEp13. The original insert containing ARO1 was 17.2 Kb BamHI fragment which complemented both nonsense and missense alleles of ARO1 in yeast. In this study, subcloning of the 17.2 Kb ARO1 fragment into pBR322, using Sau3AI partial digestion, further located the ARO1 segment to a 4 Kb to 6 Kb region. Subsequent restriction digest mapping and subcloning of the ARO1 6.5 Kb fragment with BamHI linkers led to a family of increasingly larger subclones from a common BamHI end: 1.1 Kb, 2.0 Kb, 2.9 Kb, 3.5 Kb, 4.3 Kb, 5.4 Kb, and 6.0 Kb. By using this family of subclones and a yeast ARO1 nonsense mutant, complementation analysis demonstrated that the ARO1 structural gene was located within the 3.5 Kb fragment. The 3.5 Kb fragment also complemented aroA, aroB, aroD, and aroE mutants of E. coli. Preliminary DNA sequencing studies and RNA/DNA hybridization studies have indicated the direction of transcription along the gene fragment.

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LIST OF ABBREVIATIONS

A_{600}	Optical density at 600 nanometers
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CIAP	Calf-intestinal alkaline phosphatase
CMT	100 mM calcium chloride 10 mM Tris·Cl pH 7.8 5 mM magnesium chloride (for making competent E. coli cells)
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetate (disodium salt)
Kb	Kilo base pairs
λ	Bacteriophase lambda
N	Nucleotide(s)
PABA	Paraaminobenzoic acid
PCI	Phenol-chloroform-isoamylalcohol (25:24:1)
PHBA	Parahydroxybenzoic acid
rpm	Revolutions per minute
RT	Room temperature (≈25°C)
S_1	Restriction endonuclease S_1
SDS	Sodium dodecyl sulfate
T4	Bacteriophage T4
TE	10 mM Tris·Cl pH 8.0, 1 mM EDTA
UV	Ultraviolet light

CHAPTER I

INTRODUCTION

The term "cluster-gene" was proposed by Gaertner and Cole during their studies on the "arom aggregate" in Neurospora crassa in an attempt to distinguish between those gene systems that were true clusters of contiguous, separate, tightly linked genes (gene-clusters) and those gene systems which typically contained a single gene, which encoded a single polypeptide with discrete multiple functions (cluster-genes) (22). The early observations and tentative conclusions, concerning the organization of "cluster-genes," were based upon biochemical and genetic evidence that was slowly undermining the concept of one gene/one polypeptide/one function. Although definitive evidence was unavailable in support of the cluster-gene hypothesis, it was becoming obvious that the previously studied "arom aggregates," in all fungal systems studied, appeared to be "cluster-genes"--single genes encoding multifunctional polypeptide chains.

Prior to the formulation of the cluster-gene model (1), the genetic studies of Giles (24), Giles and Case, and Case et al. (13, 12) showed that the "arom aggregate" of Neurospora crassa encoded the five enzymes of the polyaromatic biosynthetic pathway. Also, they were able to isolate mutants that lacked individual activities which mapped in discrete, nonoverlapping segments of the arom region. In addition, pleiotropic mutants of Neurospora crassa were

isolated which lacked all five activities of the arom aggregate, and these mutations typically mapped at one end of the gene. From these studies, Giles et al. concluded that the "arom gene" system was acting as a polycistronic unit of transcription (23), in direct contrast to the monocistronic, single transcriptional cluster-gene model (22).

However, biochemical studies lent support to the concept of the "cluster-gene." The five enzyme activities known to be associated with the "arom aggregate" of Neurospora crassa were shown to sediment together in sucrose gradients (24, 11). Later studies by Gaertner and Cole (22) and Lumsden and Coggins (34), using protease inhibitors and denaturing polyacrylamide gels, demonstrated that the basic subunit of the arom aggregate of Neurospora crassa was a single pentafunctional polypeptide chain. Thus, the "arom multienzyme complex" of Neurospora crassa became a classical representative of a "cluster-gene." It has since been found that analogous "arom complexes" exist in all other fungi that have been studied (2, 1), including Ustilago violacea (5), and Schizosaccharomyces pombe (45), and also in the photosynthetic organism of Euglena gracilis (6).

Unfortunately, the molecular organization of the "cluster-genes" was unknown, and definitive end-terminal and amino acid sequence analysis had not been done. However, using recent advances in molecular recombinant technology, Donahue et al. cloned, sequenced, and subsequently demonstrated the "cluster-gene" character of the HIS4 region of Saccharomyces cerevisiae (19). The HIS4

region has been shown to encode the enzymes for the second, third, and tenth reactions in histidine biosynthesis within a single trifunctional polypeptide (21). Thus, recombinant technology makes it possible to stringently examine the "cluster-gene" model proposed for other gene systems including the "arom aggregates" of the fungal systems (2, 1, 5, 45).

In the yeast Saccharomyces cerevisiae, the ARO1 gene is thought to be a "cluster-gene" encoding a single pentafunctional protein which catalyzes five steps in the central pathway leading to the biosynthesis of the aromatic amino acids (Larimer and Gaertner, manuscript in preparation; see Figure 1.1). Recently, in an attempt to test the ARO1 "cluster-gene" model further, Larimer et al. have reported the molecular cloning of a 17.2 Kb BamHI fragment from Saccharomyces cerevisiae which, according to biochemical and genetic evidence, contains the entire ARO1 gene system. The ARO1 fragment was isolated by complementation in Saccharomyces cerevisiae following transformation with a comprehensive yeast DNA library of BamHI restriction fragments inserted into the shuttle-vector YEp13 (see figures on pages 68 and 69). The 17.2 Kb fragment (see figure on page 28) complemented yeast nonsense and missense alleles of aro1, and aroA, aroB, aroD, and aroE mutants in E. coli (42). Also the complemented yeast aro1 mutants, transformed with the ARO1 containing episome, demonstrated the 150,000 dalton ARO1 gene product on polyacrylamide gels, and expressed five to twelve times the normal levels of the five ARO1 enzyme actitivites (31).

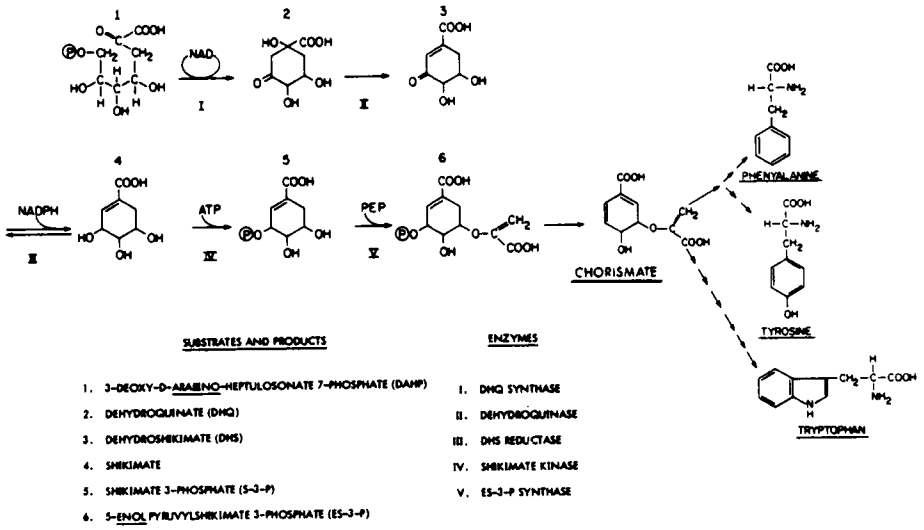


Figure 1.1. AROl biochemical pathway.

In this dissertation, I have used DNA sequencing techniques and recombinant technology (36, 29) to subclone the 17.2 Kb ARO1 fragment described by Larimer et al. (31) in an effort to characterize and define the eukaryotic ARO1 gene of Saccharomyces cerevisiae. Subcloning of the 17.2 Kb ARO1 fragment led to a family of increasingly larger subclones from a common BamHI end. By using this family of subclones and aro mutants of yeast and of E. coli, the ARO1 gene was located within a 3.5 Kb fragment. A yeast promoter was shown to be most likely responsible for the expression of the ARO1 gene in both yeast and in E. coli. Preliminary DNA sequencing and RNA/DNA hybridization studies suggest that transcription initiation originates within a region proximal to one end of the 3.5 Kb fragment (see figures on pages 50 and 53). The results presented in this study have demonstrated the isolation and partial characterization of the eukaryotic ARO1 gene from Saccharomyces cerevisiae and lend support to the cluster-gene model proposed for the ARO1 gene system (31).

CHAPTER II

MATERIALS AND METHODS

Strains and Media

E. coli strain HB101 (F^-, *hsdS20* (r_B^-, m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20* (Sm^r), *xyl-5*, *mtl-1*, *supE44*, λ^-) was used as a host during transformation and for plasmid amplification (8). *E. coli aro* strains used in transformation assays were SK3337 (F^- *gal lacy mtl xyl his proA thi aroA* λ^-), SK890 (F^- *gal thi aroB str ton hsdR4 endA sbcB15*), SK2881 (F^- *gal lac srl leuC aroD6 tonA hsdR4 recA1 sbcB15 endA*), and SK484 (F^- *gal thi aroE spc hsdR4 endA sbcB15*). These *E. coli aro* strains were the generous gift of Dr. Sidney R. Kushner, The University of Georgia, Athens.

The *Saccharomyces cerevisiae* yeast strains XL39-4A (*a aro1D leu2-3 his4-38*), XL61-1D (*aro1E ura3-52 leu2-3 his3-Δ1*), and XL60-2B (*aro1D ura3-52 leu2-3 leu2-112 thr1 arg4-1*) were constructed by Dr. Frank Larimer, Biology Division, Oak Ridge National Laboratories. These strains were used as hosts for yeast transformations. The wild type *Saccharomyces cerevisiae* yeast strain S288C (*α gal-2*) was used as a source for *ARO1* mRNA and for the *ARO1* gene.

L-broth (8) contained 5 g of sodium chloride, 10 g of bacto-tryptone, and 5 g of yeast extract per liter. The solution was adjusted to pH 7.5 with 0.5N sodium hydroxide. L-broth agar contained 1.5% bactoagar in addition to the ingredients given above. Selectable L-broth agar contained either 25 μg/ml of ampicillin or 12

µg/ml of tetracycline. The ampicillin and tetracycline were dissolved in dimethyl sulfoxide (DMSO) in final concentrations of 25 mg/ml and 12 mg/ml respectively, and stored at -20°C. The ampicillin or tetracycline was added to autoclaved medium after first cooling the medium to 55°C in a water bath. One liter of minimal defined aro⁻-broth for growing E. coli, contained 40 mls 25x Davis salts (25x Davis salts, per liter: 25 g magnesium sulfate, 12.5 g sodium citrate, 75.0 g monobasic potassium sulfate, 175.0 g dibasic potassium sulfate, 25 g ammonium sulfate), 5 g D-glucose, 20 µg/ml of all of the amino acids except the aromatic amino acids, 0.02 µg/ml of parahydroxybenzoic acid (PHBA), 0.02 µg/ml paraaminobenzoic acid (PABA), and 20 µg/ml of any necessary marker supplements. The Davis salts and the glucose were autoclaved for 15 minutes and cooled to 55°C in a water bath before the addition of filter sterilized stock amino acids, PHBA and PABA and marker supplements. Minimal defined aro⁻ broth agar contained 2.0% bactoagar in addition to the ingredients mentioned above.

SD-broth (33) was a minimal defined broth for growing yeast, and contained 7 g Difco yeast nitrogen base (without amino acids) and 20 g dextrose per liter. Minimal defined SD-broth agar contained 2.0% bactoagar in addition to the ingredients mentioned above. Sterilization was by autoclaving for 15 minutes. Any necessary marker supplements were either sterilized by autoclaving or by filter sterilization and were added to cooled (55°C) broth or agar medium to final concentrations of 20 µg/ml. These yeast media used in growth, selection, and transformation have been described (3, 33).

Plasmids

Throughout the cloning and subcloning of the ARO1 gene of Saccharomyces cerevisiae, I used two very common recombinant plasmids pBR322 and YEp13. pBR322 is an E. coli plasmid (9, 48) and YEp13 is a hybrid shuttle vector capable of stable, episomal transformation of either an E. coli or yeast host (see Figure A.1, page 67) (10, 27).

Alcohol Precipitations

Unless otherwise stated in the text, all small volume (less than 300 µl) DNA precipitations were carried out in 1.5 ml Eppendorf tubes according to the following protocol--after bringing the aqueous DNA phase to 0.3 M sodium acetate (pH 5.2) in a total volume of 400 µl, 1 ml of 95% ethanol was added; and after a 10 minute incubation at -80°C, the tube was centrifuged in a Beckman microfuge (120000xg) for 15 minutes. The supernatant was carefully withdrawn; the pellet was rinsed with 1 ml of 80% ethanol and briefly lyophilized (5-7 minutes) before final resuspension in sterilized TE (10 mM Tris·Cl pH 8.0, 1 mM EDTA).

Enzymes and Chemicals

All restriction enzymes were obtained from either Bethesda Research Laboratories or New England Biolabs. T4 RNA ligase, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, lyticase, and HindIII Lambda-DNA came from Bethesda Research Laboratories. Calf-intestinal alkaline phosphatase was from Boehringer Mannheim.

BamHI oligonucleotide linkers were purchased from Collaborative Research Ltd. Radiolabelled compounds were from New England Nuclear. Acrylamide, urea, ampicillin, and tetracycline were from Sigma. Dimethylsulfate and hydrazine came from Aldrich Chemical Co. and Eastman Kodak, respectively. Formic acid (90%) and piperidine (10 M) both came from Fischer Scientific.

DNA Mini-Preparations

DNA mini-preparations (8) were performed as an alternative to the longer, more rigorous protocols designed to provide highly purified supercoiled plasmid DNA (7, 16, 17, 29). DNAs from mini-preparations were digested with restriction endonucleases and used in transformation assays and subcloning experiments without further purification. Single colony isolates from selective medium plates (usually L-broth-ampicillin 25 µg/ml) were inoculated into sterile 50 ml Erlenmeyer flasks containing 20 ml of L-broth plus 25 µg/ml ampicillin. The flasks were incubated overnight in a 37°C water-bath shaker at 250 rpm. The following day, the flask was cooled on ice for 15 minutes; after which the cells were spun down in 30 ml Oak Ridge centrifugre tubes in an appropriate Sorvall rotor at 5000 rpm for 5 minutes at 4°C. The supernatant was discarded; the cells were then resuspended in 5 ml of TE buffer (10 mM Tris·Cl pH 8.0, 1 mM EDTA) by vigorous vortexing, and transferred to 10 ml Oak Ridge tubes. Freshly made lysozyme (10 mg/ml in TE buffer) was added to a final concentration of 300 µg/ml, and the tube incubated on ice for 20 minutes. The cells were frozen solid in liquid nitrogen and

and thawed quickly in a 37°C water-bath. The cell mixture was diluted with 2.5 ml of cold TE buffer and vortexed vigorously for 1 minute. Cell debris was separated from the plasmid containing supernatant by centrifugation in a Beckman 50Ti rotor at 20,000 rpm for 30 minutes at 4°C. The supernatant was poured from the debris pellet into glass Corex tubes (15 ml) and immediately brought to 0.1 M sodium chloride, 0.1% sarkosyl and phenol-extracted two times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The aqeuous phase was removed to a fresh 15 ml Corex tube and ether-extracted three times to remove phenol. The aqueous phase (3-4 ml) was then brought to 0.3 M sodium acetate and alcohol precipitated with the addition of 2.5 volumes of cold absolute ethanol, incubated at -80°C for 30 minutes, and followed by centrifugation in an appropriate Sorvall rotor at 10,000 rpm for 30 minutes. The supernatant was discarded and the pellet rinsed with 80% ethanol before a brief lyophilization (3-5'). The pellet was resuspended in 150 µl of TE buffer and stored at 4°C in 1.5 ml Eppendorf tubes. The yield of plasmid DNA from a 20 ml overnight culture was usually 10-15 µg (estimated using comparisons with known amounts of standard DNA run on 0.7% agarose gels).

Phosphatase Reactions

The terminal 5' phosphate groups were removed from purified DNA samples using calf-intestinal alkaline phosphatase (CIAP) (14, 44, 50). The phosphatase reaction was carried out in a total reaction volume of 100 µl containing CIAP buffer (50 mM Tris·Cl pH 9.0, 1 mM magnesium chloride, 0.1 mM zinc chloride, 1 mM spermidine

hydrochloride), 1-60 µg of DNA, water, and 0.3 U of CIAP. The reaction mixture was incubated in a 37°C water-bath overnight. After overnight incubation at 37°C, a second aliquot of CIAP (0.3 U) was added followed by an additional 6 h of incubation in a 37°C water-bath. Two successive phenol extractions, followed by two extractions with ether, an alcohol precipitation, and two pellet washes using 80% ethanol (RT) left the DNA suitable for subsequent restriction analyses, 5' or 3' end labelling, or ligation.

Ligations

Any plasmids capable of self-ligation during subcloning construction were first digested with the appropriate restriction endonuclease and treated with alkaline phosphatase, followed by electroelution of the linearized plasmid from preparative 0.7% agarose gels. The efficiency of the phosphatase treatment was tested, after overnight ligation of the plasmid, in transformation assays with competent E. coli HB101 cells. When the test transformation, using 5.0 µg of plasmid, produced fewer than 1% of the colonies produced by 5.0 µg of plasmid that had not been treated with CIAP, the treated plasmid was alcohol precipitated and resuspended in TE buffer (10 mm Tris·Cl pH 8.0, 1 mm EDTA) at 10 µg/µl. Plasmids to be used in subcloning experiments that were not capable of self-ligation were generated with the appropriate restriction endonucleases, purified from preparative 0.7% agarose gels, and resuspended at 10 µg/µl in TE buffer (e.g., plasmids containing two different restriction-site termini).

Insert fragments for subcloning were electroeluted from 0.7% agarose gels and resuspended at 10 µg/ml in TE buffer.

Ligation reactions were carried out in a 16°C water-bath for 18 h in a total volume of 20 µl composed of standard T4 DNA ligase buffer (50 mM Tris·Cl pH 7.8, 10 mM magnesium chloride, 20 mM dithiothreitol, 1 mM ATP, 50 µg/ml BSA), water, T4 DNA ligase (400 U), and a plasmid-to-insert molar ratio of 1:4 (molecule:molecule), with an upper limit of approximately 200 µg total nucleic acid per 20 µl reaction. In most cases, the general scheme stated above produced the desired recombinant products; however, a more detailed protocol for maximizing the ligation products between plasmid and insert was followed as described (20).

Linkers were ligated to DNA fragments at 16°C for 24 h in a total reaction volume of 20 µl typically containing T4 DNA ligase buffer, water, T4 DNA ligase (400 U), T4 RNA ligase (2 U), and a 100 fold molar excess of linker to fragment--and usually contained very high total nucleic acid concentrations (1.0-10 mg/ml) (37). The linker reaction was then digested for 24 h at 37°C in a 100 µl overnight reaction in the appropriate restriction endonuclease buffer, water, and 1 U of restriction enzyme per microgram of DNA to remove excess linker. Fragments containing linkers were purified from 0.7% agarose gels and resuspended at 10 µg/µl--ready for subcloning into purified vector.

Curing Experiments

Curing experiments were performed after transformation in an effort to demonstrate that a particular phenotype being expressed

was actually due to the transformation event (uptake and expression of plasmid DNA) and not due to reversion or mutation phenomena within a given mutant. A transformed colony was picked from selective agar medium (e.g., aro minus medium) and grown for 24 h in rich non-selective broth (L-broth for E. coli and YEPD for yeast). During the 24 h growth period under non-selective conditions, many cells would be "cured" of the plasmid. The efficiency of curing was approximated by plating equal amounts of the 24 h culture on selective and non-selective agar medias. The ratio of the number of colonies on selective agar (e.g., ampicillin resistant colonies) to the number of colonies on non-selective agar (e.g., L-broth agar colonies) gave an approximation of the percentage of cells "cured" within a 24 h time period grown under non-selective conditions. By streaking colonies from the non-selective plate (e.g., L-broth agar colonies) onto both L-broth agar and L-broth agar plus ampicillin plates, colonies lacking plasmid ("cured" colonies) could be isolated. These "cured" colonies were then back-streaked on selective plates (e.g., aro minus media) to insure that the ability to grow on a minimal defined media was due to the uptake of plasmid DNA and not due to reversion or mutation phenomena.

Bacterial Transformation

Bacterial strains for transformation were made competent according to the protocol below (8, 35). A single colony isolate was inoculated in 20 ml of L-broth and grown at 37°C overnight in a water-bath shaker. The following day, a 560 µl aliquot of the

overnight culture was added to 56 ml of L-broth in a 250 ml side-arm flask (1 cm pathlength). The culture was then grown to an A_{600} of 0.16 and the flask placed in an ice-water bath for 10 minutes. The cells were spun down in sterile 30 ml Oak-Ridge tubes, resuspended in 28 ml of CMT buffer (0.1 M calcium chloride, 5 mM magnesium chloride, 10 mM Tris·Cl pH 7.8), and incubated on ice for 25 minutes. The cells were spun down again and resuspended in 1.4 ml of CMT buffer. The transformation procedure was begun by the addition of DNA to the competent cells in a ratio of 5-10 µl of aqueous DNA (0.01 µg-10 µg) to 200 µl of competent cells and incubated on ice for 15 minutes. Following a 5-minute heat shock in a 37°C water-bath, 800 µl of L-broth was added per 200 µl transformation aliquot and the mixture vigorously shook in a 37°C water shake-bath for 60 minutes. The transformation aliquot was plated on selective medium or amplified directly as described elsewhere in this thesis.

Yeast Transformation

The transformation protocol followed was a slight modification of the yeast transformation procedure outlined by Ito et al. (30). Basically, an overnight starter culture was begun by inoculating an isolated yeast colony in 20 ml of YEPD. The culture was grown in a 30°C water-bath with continuous shaking (250 rpm). The following day, 200 µl of the starter culture was inoculated into 200 ml of YEPD and grown in a 30°C water-bath with shaking (250 rpm) until the A_{600} reached 0.1 (1 cm pathlength (5×10^6 cells/ml)). The cells were then pelleted in sterile 250 cc Sorvall bottles by centrifugation

at 5000 rpm for 10 minutes at 4°C. The pellet was washed with 10 ml of TE (10 mM Tris·Cl 8.0, 1 mm EDTA), transferred to sterile 50 cc Falcon tubes, repelleted, and washed once with 10 ml of lithium acetate-TE (100 mM lithium acetate, TE pH 7.5), before a final resuspension in 1 ml of lithium acetate-TE pH 7.5. The culture was shook in a 30°C water-bath at 100 rpm. After 30 minutes, 100 µl aliquots were put into 1.5 ml Eppendorf tubes along with 10 to 50 µl of TE that contained up to 10 µg of DNA. The transformation mixture was incubated without shaking for 30 minutes at 30°C; after which 700 µl of lithium acetate-TE that contained 40% polyethylene glycol was added before an additional incubation, without shaking, at 30°C for 60 minutes. After the 60 minute incubation, the cells were heat-shocked at 42°C for 5 minutes and pelleted in a Beckman microfuge for 5 minutes. The supernatant was decanted, the cell pellet was resuspended in 1 ml of YEPD, and shook in a 30°C water bath for 60 minutes. After 60 minutes, the cells were repelleted, resuspended in 100 µl of sterile water, and surface plated onto the appropriate selective medium.

Large Scale Preparation of Plasmid DNA

A starter culture was prepared from an overnight-grown, single colony inoculum, of plasmid containing *E. coli* HB101 or *E. coli* SK2881 in L-broth with the appropriate drug added (25 µg/ml ampicillin or 12 µg/ml tetracycline) (7, 16, 17, 28). Ten ml of the starter culture was transferred to 500 ml of the same medium and grown to stationary phase at 37°C and 250 rpm for 18 hr. Cells

from a 500 ml culture were cooled on ice-water for 10 minutes and pelleted in 500 ml Sorvall bottles at 5000 rpm for 20 minutes at 4°C. The pellet and bottle were rinsed with cold distilled water to get rid of excess medium. The pellet was resuspended in 8 ml of 50 mM glucose, 25 mM Tris·Cl pH 8.0, and 10 mM EDTA. Freshly made lysozyme in TE was added to the cells to a final concentration of 300 µg/ml. The culture was then transferred to an SW28 polyallomer tube and incubated at room temperature for 5 minutes. Then, 18 ml of freshly made 0.2 N sodium hydroxide and 1% SDS were added to the suspension. The tube was covered with parafilm and the contents mixed by gently inverting the tube several times. After incubating the tube on ice for 10 minutes, 12 ml of an ice-cold solution of 5 M potassium acetate pH 4.0 (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of water) was added, mixed, and again the tube was incubated on ice for an additional 10 minutes. The lysed cell suspension was centrifuged in a Beckman SW28 rotor at 20,000 rpm for 20 minutes at 4°C. Cell DNA and bacterial debris formed a tight whitish pellet. The supernatant, approximately 36 ml, was transferred to 150 ml Corex bottles. Plasmid DNA was precipitated with the addition of 0.6 volumes of isopropanol at room temperature for 15 minutes. The DNA was recovered by centrifugation in an appropriate Sorvall rotor at 5000 rpm for 30 minutes at room temperature. The supernatant was discarded, and the pellet was rinsed twice with 20 ml of 80% ethanol by rolling the alcohol around the bottle and pellet. The Corex bottle was drained and briefly dried in a vacuum

dessicator (≃5 minutes). Each pellet from a 500 ml culture was resuspended by aspiration with 15 ml of TE. In preparation for cesium chloride equilibrium centrifugation, 1 g of cesium chloride and 70 µl of 10 mg/ml ethidium bromide was added per ml of TE used to resuspend the plasmid pellet. The DNA suspension was transferred to Beckman 50Ti polyallomer tubes and centrifuged in a Beckman 50Ti rotor at 35,000 rpm for 60 h at 20°C. This procedure (43) repeatedly gives two distinct bands of DNA: an upper relaxed band of bacterial DNA and nicked circular plasmid DNA, and a more dense, lower, supercoiled band of covalently closed, circular DNA. Both DNA bands could more easily be visualized using a hand-held long-wave UV light. The supercoiled DNA was easily removed using the following procedure: The tube was supported and wrapped with clear tape around the region of the supercoiled band. A puncture was made approximately 3 mm below the supercoiled band with a #21 gauge needle and 3 ml syringe. With the bevel of the needle facing up, the supercoiled band was gently removed. The pooled DNA was extracted with water-saturated n-butyl alcohol until both phases were clear. The aqueous DNA phase was diluted to 50 ml (≃5X) with TE, brought to 0.3 M sodium acetate pH 5.2, and alcohol precipitated with the addition of 2.2 volumes of cold 95% ethanol after incubation in a -20°C freezer for at least 1 hr in 150 ml Corex bottles (need two bottles). The DNA pellet was rinsed with 20 ml of 80% ethanol, briefly lyophilized, and resuspended in 2 ml of TE. The DNA was filtered through a 2 cm x 25 cm Biogel A5m column previously equilibrated with TE. The DNA was eluted with TE

at an elution rate of approximately 0.5 ml/minute. The DNA-containing fractions were determined using an in-line UV-recording system (absorbance at 260 nm). The DNA fractions were pooled and alcohol precipitated in SW28 tubes as noted previously. DNA prepared in this way was free of bacterial RNA and protein and was suitable for restriction enzyme analyses and DNA sequencing studies. The yield of supercoiled DNA from a 500 ml culture was consistently 150 to 200 µg. The DNA was stored in 1.5 ml Eppendorf tubes at 4°C and used in all subsequent procedures without further purification.

Restriction Mapping

All restriction digests were performed according to the protocol sheets which accompany the commercial products. Restriction fragment molecular weights were calculated using either HindIII digested lambda DNA or HinfI-digested pBR322 marker DNAs which were run in parallel with the unknown DNAs in either 0.7 agarose gels or 5% polyacrylamide gels (1:30 bisacrylamide:acrylamide). The restriction sites within a given fragment were determined using the multiple digestion approach of Lawn et al. (32).

Fragment Elution from Agarose Gels

All DNA fragments were purified from agarose gels by electroelution into dialysis bags (40). DNA fragments of interest were localized in agarose gels after staining the gel in TBE buffer (90 mM Tris-borate pH 8.3, 25 mM EDTA) containing 1 µg/ml of ethidium bromide for 15 minutes at room temperature, using a long-wave UV

lamp to minimize damage to the DNA. The fragment was excised from the agarose slab using a razor blade; the cuts were made to within 1 mm of the band. The excised agarose fragment-slice was then inserted into an 0.6 mm dialysis bag approximately 3 cm longer than the slice. The bag was then filled with the same TBE buffer, the air bubbles expelled, and the bag clipped within 1 cm of the agarose fragment slice. The fragment was electroeluted from the agarose slice at 100V for 2-3 hours (depending upon the size). The band was usually visible under long-wave UV light as a dense packed band against the side of the dialysis tubing. The current was not reversed to pull the DNA off the dialysis bag, because I found this step to be unnecessary. The bag was rolled between thumb and forefinger to release the loosely bound DNA, and the fluid was carefully removed. If the volume was greater than 400 µl, sec-butanol was used to concentrate the aqueous phase to 400 µl, followed by two ether extractions (1 ml per extraction) and a standard alcohol precipitation.

Fragment Elution from Polyacrylamide Gels

DNA molecules which had been 5' end labeled were separated on 5% polyacrylamide gels (1:30 bisacrylamide to acrylamide) using Tris-borate-EDTA (TBE) buffer (90 mM Tris·borate pH 8.3, 25 mM EDTA). The gels were electrophoresed at 200V for approximately 2 h. pBR322 DNA cut with HinfI was used as size marker DNA. The bands were visualized under long-wave UV irradiation after staining in ethidium bromide (1 µg/ml) for 15 minutes at room temperature. The appropriate bands were excised from the gel with a razor blade and crushed to a paste

with a teflon pestle in a 1.5 ml siliconized Eppendorf tube. The DNA was eluted from the gel (38) slurry overnight in a 37°C water bath with 600 microliters of DNA extraction buffer (DEB) (0.5 M ammonium acetate, 5mM magnesium acetate, 1 mM EDTA, 0.1% SDS). The next day, the gel slurry was centrifuged at 12,000xg for 5 minutes in a Beckman microfuge, and the supernatant was removed to a fresh 1.5 ml Eppendorf tube. For fragments under 1 kilobase in size, this elution procedure standardly removed 90% or more of the total counts available. The supernatant was then cleared of any remaining gel particles by microfuge centrifugation at 12,000xg for 5 minutes; after which, all but 5 microliters was transferred to a clean, siliconized 1.5 ml Eppendorf tube. DNA (approx. 500 µl) was then alcohol precipitated with 40 µg of yeast t-RNA. The pellet was re-suspended in 300 µl of 0.5 M ammonium acetate and precipitated with 800 microliters of absolute ethanol. The ammonium acetate precipitation was repeated, and the pellet rinsed two times with 80% ethanol (1 ml/rinse). The pellet was lyophilized to near dryness and re-suspended by vigorous vortexing in 47 microliters of sterile glass distilled deionized water. At this point, the DNA was ready for the Maxam-Gilbert sequencing reactions (38).

5' Labelling of 5' Extensions

All labelling reactions began with approximately 60 µg of the appropriate DNA purified and amplified as described elsewhere in this thesis. Appropriate restriction endonuclease digestion of the DNA

was done in 1.5 ml Eppendorf tubes in a 37°C water-bath overnight as described in 100 µl reaction volumes. Before proceeding with the phosphatase reaction, the DNA was run on a mini-gel to check the extent of the primary restriction digestion. The DNA was neither phenol extracted or alcohol precipitated before the phosphatase reaction was begun. The original 100 µl restriction volume was brought to 200 µl total using standard 10X calf intestinal alkaline phosphatase (CIAP) buffer and 0.25U of CIAP, and the reaction mixture was incubated at 37°C overnight. The following day, a second aliquot (0.25U) of CIAP was added and an additional incubation at 37°C for 4-6 h was performed. The reaction mixture (≃225 µl) was extracted twice with phenolchloroform-isoamyl alcohol (PCI) (25:24:1) and then ether extracted twice (1 ml per extraction). The phenol phases were re-extracted with an equal volume (equal to the original aqueous volume) of water and the aqueous phases were combined (≃500 µl) and alcohol precipitated. The pellet was rinsed twice with 1 ml of 80% ethanol and briefly lyophilized (≃5-8 minutes). The pellet was resuspended in 44 µl of aqueous γ-[^{32}P]ATP (3000 Ci/mmole) with vigorous vortexing. The reaction volume was brought to a total of 50 µl using standard polynucleotide kinase buffer (70 mM Tris·Cl pH 7.6, 100 mM potassium chloride, 10 mM magnesium chloride, 5 mM dithiothreitol), water, and T4 polynucleotide kinase (12U-20U per reaction) (39). The reaction was carried out at 37°C for 90 minutes, and was terminated by alcohol precipitation of the DNA. Since both 5' termini were now labelled, a second appropriate restriction enzyme

digestion was performed in a 50 µl reaction volume to generate two separable fragments each with only one end labelled. These fragments were then separated and purified from 5% polyacrylamide gels (1:30 bisacrylamide:acrylamide) as described.

5' Labelling of 3' Extensions and Blunt Termini

The basic labelling protocol used to label the 5' termini of DNA fragments containing either 3' extensions or blunt termini was identical to the procedure used for labelling 5' termini with 5' extensions except for the following: after the appropriate restriction digestion, the DNA was alcohol precipitated and resuspended in 50 µl reaction volume containing standard T4 DNA polymerase buffer (30 mM Tris·acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 200 µg/ml BSA), water, and 15U of T4 DNA polymerase (41). The reaction was carried out at 37°C for 30 minutes. The DNA was alcohol precipitated, phosphatased, labelled, and purified as described for the 5' labelling of the 5' extension protocol described above.

Nucleotide Sequence Determination

DNA fragments for sequencing were single-end 5' labelled and purified as described elsewhere in this chapter. The fragments were subjected to partial chemical degradation by the method of Maxam and Gilbert (38, 39) (see Figure A.3, page 70). In every case, the junction between two adjacent sequenced regions was determined by sequencing an overlapping DNA fragment. The DNA was sequenced on 20%, 8%, 6%, and 4% polyacrylamide (1:30 bisacrylamide:acrylamide) 7 M urea

gels, which were 0.4 mm x 20 cm x 84 cm with 1.3 cm wells (20% gels were 0.4 mm x 20 cm x 40 cm). All gels were poured in a nearly horizontal position, hardened horizontally overnight, and prerun for 1 h or until the upper portion of the outer glass (8 mm plate glass) reached approximately 50°C. For 20% gels, each well (G, A + G, T + C, C) contained 5 x 10^4 cpm, and the electrophoresis was carried out at 1200 V. For all other gels, each well contained approximately 10 x 10^4 cpm, and the electrophoresis was carried out between 2100 V and 2500 V. The length of any particular gel run was determined empirically by noting positions of the xylene cyanol and bromophenol blue marker dyes and the corresponding nucleotide sizes comigrating with these dyes within a given gel type. After a gel run, one glass plate was removed, and the gel strip was covered with one layer of Saran-wrap and strips of Kodak XAR-5 film. The top glass plate was replaced, and the entire assembly was wrapped in a light-tight cloth cassette. The gels were exposed to film at -80°C for 1-4 days without intensifying screens before development with standard Eastman Kodak chemicals.

Isolation of RNA

RNA was isolated from the wild type yeast strain S288C (α, gal-2). The protocol followed was a combination of two protocols outlined by Struhl and Davis (46, 47) and by Chirgwin et al. (15). Essentially, a starter culture was prepared by inoculating 20 µl of a stationary YEPD broth culture of S288C into 20 ml of SD broth with shaking in a 30°C air shake-bath at 250 rpm overnight. The following

day, 400 µl of the starter culture was inoculated into 500 ml of sterile, 30°C SD broth. The culture was grown in a 30°C air shake-bath at 250 rpm until the A_{600} reached 0.15 using a 1 cm pathlength. The cells were pelleted in 500 ml Sorvall bottles at 5000 rpm for 30 minutes. The pellet, approximately 1.5 g, was washed with 10 ml of water, repelleted and quickly resuspended by aspiration in 1 ml of room temperature 1 M sorbitol containing 50 mM sodium phosphate buffer pH 7.5. After resuspension, 3U of lyticase per original ml of cell culture was added, and the culture was lightly vortexed. Following a 5 minute incubation at room temperature, 5 ml per gram cell pellet of guanidine thiocyanate solution (4 M quanidine thiocyanate, 0.5% sarkosyl, 25 mm sodium citrate pH 7.0, and 0.1 M 2-mercaptoethanol) was added and the solution homogenized in a sterilized, stainless-steel container using 15 second bursts four times. Cell debris was removed by centrifugation in sterilized 30 ml Oak-Ridge centrifuge tubes in an appropriate Sorvall rotor at 10,000 rpm for 10 minutes. The aqueous phase was removed and brought to a total of 8 ml using sterile water. The 8 ml resuspension was layered onto a 4 ml 5.7 M cesium chloride containing 0.1 mM EDTA in 12 ml Beckman SW41 tubes (25, 26). The RNA was pelleted by centrifugation, the top aqueous phase and lower cesium phase was drawn off with a Pasteur pipette until the obvious regions of debris and DNA were discarded; after which, the remainder of the solution was poured off and the tube was drained. The pellet was rinsed two times with 1 ml of 80% ethanol, dried briefly, and resuspended in 0.5 ml of sterile water by incubation at 68°C for 30 seconds. The

RNA was transferred to a sterile 1.5 ml Eppendorf tube, alcohol precipitated and resuspended in 0.5 ml of sterile water. The amount of RNA was quantitated by checking the absorbance at 260 nm using a 1 cm pathlength ($A_{600} \simeq 40$ µg/ml). The RNA was used for dot blot analysis.

Dot Blot Analysis

Dot blot analysis was performed using a slight modification of two protocols outlined by Thomas (49) and White and Bancroft (51). Purified RNA in sterile water was mixed with an equal volume of a solution containing 2 parts 37% formaldehyde and 3 parts 20x SSC (20x SSC: 3 M sodium chloride, 0.3 M sodium citrate). The solution was heated in a 60°C water-bath for 20 minutes and quickly cooled in an ice/ethanol bath. Additional 20x SSC was added to bring the final salt concentration to 15x SSC. Serial dilutions were carried out such that the RNA content of each dot was decreased but the volume added to the filter was constant; thereby, each dot was approximately the same size on the filter. The filter was dried at room temperature for 60 minutes and baked in a vacuum oven at 80°C for 3 h. The filter was placed in a Sears seal-a-meal bag containing 0.12 ml/cm^2 hydridization solution (hybridization solution: 1:1 2x hybridization stock to deionized, filtered formamide pH 7.0; 2x hybridization solution: 10x SSC, 100 mM sodium phosphate pH 6.5, 500 µg/ml denatured salmon sperm DNA, and bovine serum albumin, Ficoll, and polyvinylpyrrolidone at final concentrations of 0.4%). The filter was prehybridized in hybridization solution for 24 h in a 42°C

water-bath; the prehybridization solution was removed and a fresh aliquot of hybridization solution containing 10% dextran sulfate and 10^6 cpm/75 cm^2 of the appropriate [^{32}P]-DNA probe. The hybridization was carried out for 48 h in a 42°C water-bath. The probe was 5' labelled DNA according to the protocol given elsewhere. Before probe was added to the hybridization solution, the probe was diluted with 10 volumes of hybridization solution and denatured in a boiling water-bath for 10 minutes. After hybridization, the filter was removed from the bag and placed in a plastic pan for rinsing. The initial rinses were repeated four times with 2x SSC containing 0.1% sodium dodecyl sulfate (SDS) at 55°C, 15 minutes per rinse. The second set of rinses were identical to the first, except that the saline concentration was reduced to 0.2x SSC. The final four rinses were done at room temperature with 0.2x SSC without SDS. The filter was drained on blotting paper and dried at room temperature for 60 minutes, placed in a Sears seal-a-meal bag and exposed to Kodak XAR-5 film for 24 h at -80°C prior to development of the autoradiogram.

CHAPTER III

RESULTS

Isolation of the 17.2 Kb ARO1 Fragment

The isolation and characterization of the original 17.2 Kb ARO1 cluster-gene fragment has been described (31). The original fragment was isolated by complementation in Saccharomyces cerevisiae following transformation with a comprehensive yeast DNA library of BamH1 restriction fragments inserted into the shuttle-vector YEp13 (see Figure A.2, page 69). A restriction map of that fragment is shown in Figure 3.1.

ARO1 Promoter Activity

In order to demonstrate that the expression of the 17.2 Kb ARO1 fragment in the YEp13 shuttle-vector is due to a promoter on the fragment (and not due to the tetracycline promoter on the plasmid), YEp13 plasmids containing the 17.2 Kb ARO1 insert in both orientations relative to plasmid were constructed and tested for expression in both orientations. DNA from ARO1 transformed E. coli HB101 cells was amplified and purified as described. The 17.2 Kb BamH1 fragment was purified from preparative 0.7% agarose gels and ligated back into the BamH1 site of the YEp13 shuttle-vector. The ligation mixture was then used to transform competent E. coli HB101 cells. The transformation mixture was plated on L-broth agar-medium containing ampicillin (25 µg/ml) or tetracycline (12 µg/ml).

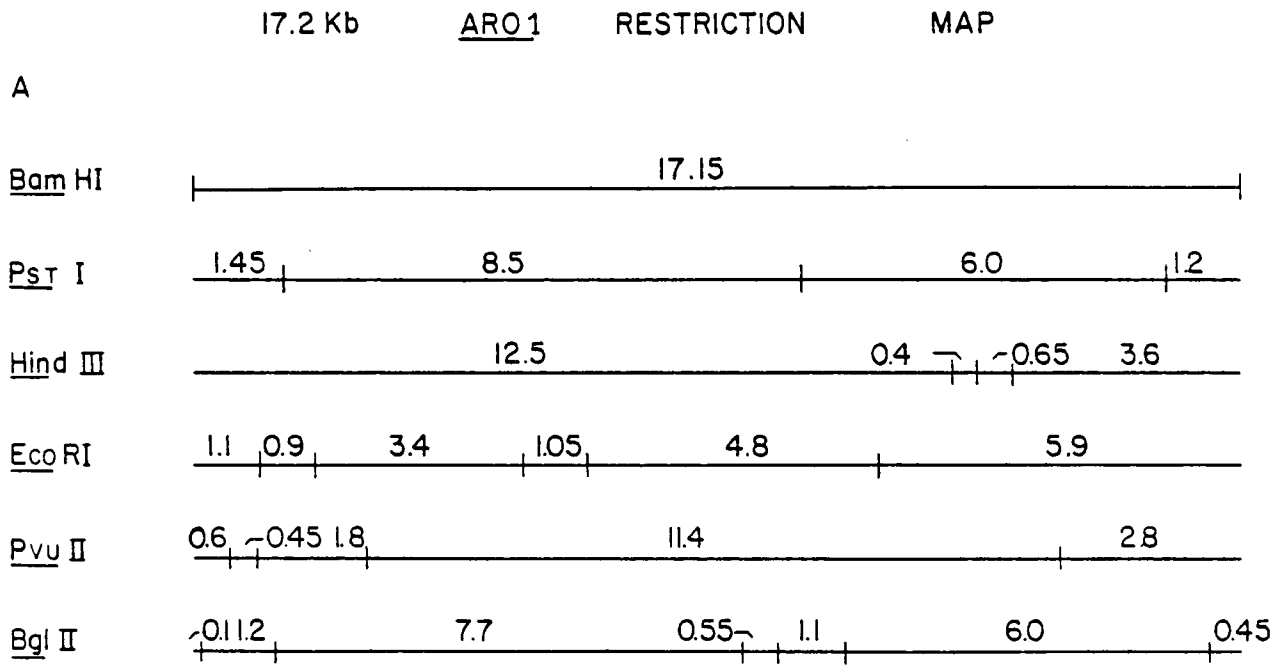


Fig. 3.1. Restriction map of the 17.2 Kb ARO1 fragment. Restriction map of the initial 17.2 Kb ARO1 fragment. The first subcloned fragment demonstrating ARO1 activity was a 17.2 Kb BamHI fragment (see text). On the left are those restriction endonucleases which have internal sites mapped within the BamHI boundaries of the 17.2 Kb fragment.

Colonies suspected of containing insert and which grew on the ampicillin plates, were picked for further analysis. DNAs from mini-preparations were digested with BamHI and HindIII restriction endonucleases and electrophoresed on 0.7% agarose gel slabs to determine the relative orientation of the 17.2 Kb fragment to the plasmid. The two distinct orientations of the 17.2 Kb fragment and the predicted fragment sizes are shown in Figure 3.2. These same two DNA preparations were used to transform aro1 mutants of yeast. Using either orientation, the 17.2 Kb ARO1 fragment was capable of complementing the pleiotropic aro1D nonsense mutant of yeast (data not shown). No differences in complementation efficiency or growth of complemented strains were observed relative to the orientation of the 17.2 Kb fragment.

ARO1 Expression in E. coli

It was of interest to determine if the yeast ARO1 gene would be expressed, and therefore complement, aro mutants of E. coli. With plasmid DNAs from subclones containing the YEp13 vector and the original 17.2 Kb ARO1 fragment in both orientations (see Results: ARO1 Promoter Activity), transformations were performed using four separate aro mutants of E. coli (see Strains and Media). In either orientation, all aro mutants of E. coli were complemented, and curing experiments demonstrated that the ARO+ phenotype was due to expression of the fragment and not due to reversion (data not shown). No differences in complementation efficiency were observed with respect to orientation.

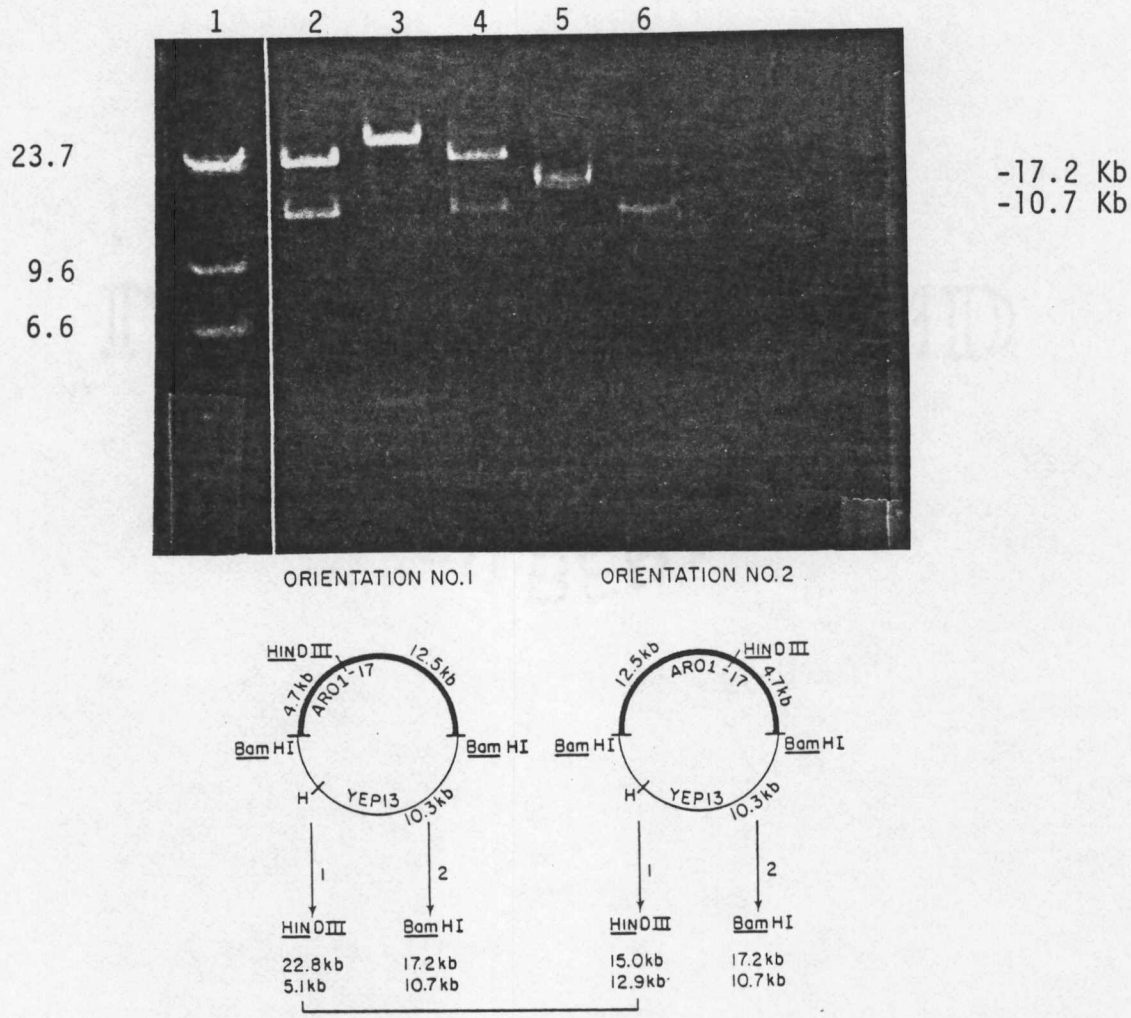


Fig. 3.2. 17.2 Kb flip-flop experiment. In order to determine whether or not the 17.2 Kb ARO1 fragment contained its own promoter activity, the 17.2 Kb fragment was purified from 0.7% agarose gels, re-ligated into BamHI restriction endonuclease digested YEp13 vector, and transformed into competent E. coli HB101 cells. DNA mini-preparations were made from selected plasmid-containing colonies, and 20 µl aliquots were restriction endonuclease digested with either BamHI or HindIII to determine orientation of the fragment relative to vector. As shown in the figure, all BamHI digestions should yield a 17.2 Kb insert fragment and a 10.7 Kb plasmid fragment. HindIII digestions will generate two different restriction patterns depending upon orientation relative to vector. In one orientation, two fragments of 23 Kb and 4 Kb will be produced, and in the other orientation, two different fragments of 14 Kb and 13 Kb will be produced. Lane 1: HindIII digested lambda DNA; Lanes 2 and 3: BamHI (2) and HindIII (3) digested 17.2 Kb DNA (orientation 1); Lanes 4 and 5: BamHI (4) and HindIII (5) digested 17.2 DNA (orientation 2); Lane 6: BamHI digested YEp13 vector DNA, 10.7 Kb.

Subcloning of the 17.2 Kb ARO1 Fragment in Yeast

The ARO1 gene has an estimated size of 3.5 Kb to 4 Kb (estimated from the ARO1 purified gene product size on denaturing polyacrylamide gels of 150,000 daltons) (31). Therefore, in an effort to locate the position of the ARO1 gene within the 17.2 Kb fragment more accurately, restriction fragments (see Figure 3.3) of at least 4 Kb of contiguous DNA were purified from 0.7% agarose gels and ligated into the YEp13 shuttle-vector. The ligation mixtures were first used to transform competent E. coli HB101 cells. DNA mini-preparations and restriction analyses were used to demonstrate the presence of the fragment being subcloned. When the correct fragment had been subcloned, aliquots of the appropriate DNA mini-preparations (1-5 μg) were used to transform the yeast, aro1D nonsense mutant. The results of these subcloning experiments and transformations are shown in Figure 3.3. Only the 12 Kb BamHI-HindIII fragment was capable of complementing the yeast mutant. Curing experiments demonstrated that the ARO+ phenotype was due to the presence of the insert in the plasmid and not due to reversion. Subsequent subcloning used a purified 12 Kb fragment.

Sau3AI Subcloning of the 12 Kb ARO1 Fragment

Partial digests of the purified 12 Kb ARO1 fragment were carried out with Sau3AI. The amount of Sau3AI necessary was determined empirically. The partial digests were electrophoresed on 0.7 agarose gels at 40 V for 18 h. HindIII digested λ-DNA was used

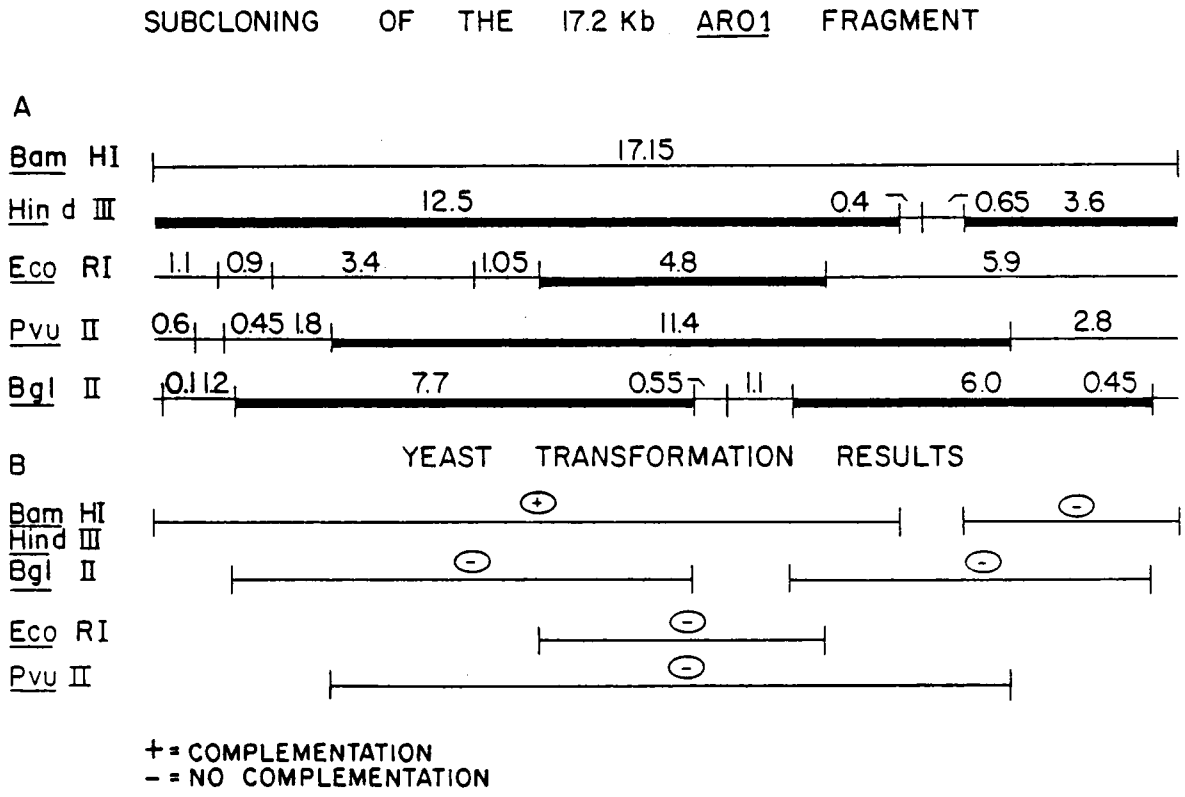


Fig. 3.3. Subcloning of the 17.2 Kb ARO1 fragment in yeast. Subcloning of the 17.2 Kb fragment was done by using complementation analysis in the yeast aro1D nonsense mutant. Selected regions of the 17.2 Kb fragment were digested with the indicated restriction endonucleases (shown in bold outline in the figure) purified on 0.7% agarose gels, and ligated into YEp13 vector. Each subcloned fragment was then tested for complementation after transformation into the yeast aro1D nonsense mutant. As indicated in the figure, the only fragment isolated which demonstrated ARO1 activity was the 12.5 Kb BamHI-HindIII fragment.

as a marker for molecular weight standards. The partial digests of the purified 12 Kb fragment were visualized with ethidium bromide staining and long-wave UV light. DNA fragments between 4 Kb and 6 Kb were electroeluted and ligated into the BamHI site of the pBR322 vector which had previously been treated with alkaline phosphatase. The ligation mixture was then used to transform competent E. coli SK2881 cells. Transformed colonies (those colonies that grew on ampicillin agar medium but failed to grow on tetracycline agar medium), were used for DNA mini-preparations and restriction analyses. However, since the Sau3AI fragments were ligated into a BamHI digested plasmid, the resultant BamHI/Sau3AI hybrid site, was no longer recognized by BamHI (Sau3AI will recognize the hybrid site, but will also destroy the insert because of the partial nature of the Sau3Al digestion used to generate the inert fragments). The size of the insert was estimated from the total length of the HindIII digested plasmid (no HindIII sites in the insert). Predicted plasmid plus insert lengths should range between 8 Kb and 10 Kb (pBR322 4.3 Kb + insert 4-6 Kb). The results of the Sau3AI subcloning experiments are shown in Figure 3.4. Because the ARO1 gene fragment seemed to be located on the left end of the 17.2 Kb fragment (see Figure 3.3), and because of the nature of the Sau3AI/BamHI ligations, it was possible to determine which of the subclones seen in Figure 3.4 contained the left-most region of the subcloned 12 Kb fragment. The rationale behind the protocol is shown in schematic form in Figure 3.5. Those subclones that had apparently regenerated the

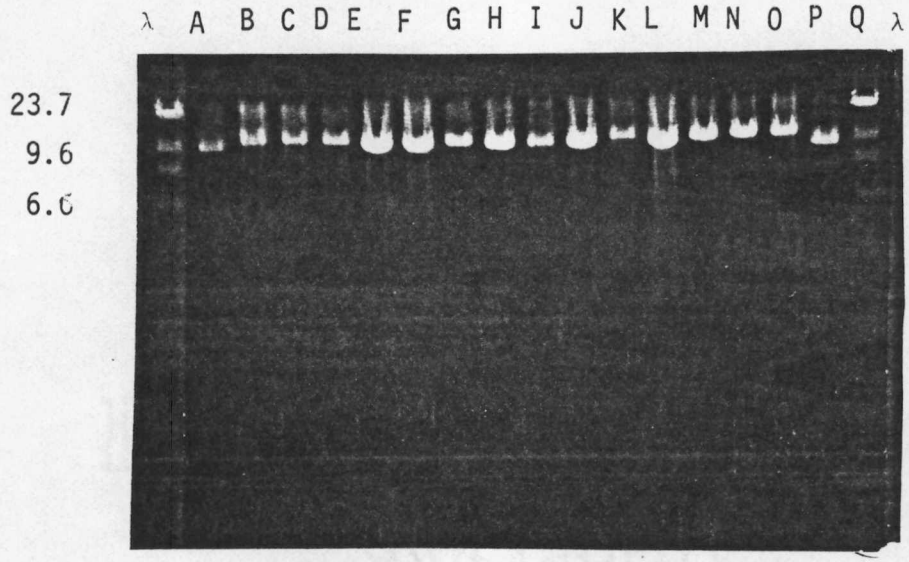


Fig. 3.4. Sau3AI subclones. The 12.5 Kb ARO1 fragment was purified from 0.7% agarose gels and partially digested with Sau3AI. Sau3AI fragments between 4 Kb and 6 Kb (see text) were electroeluted from 0.7% agarose gels and ligated into BamHI digested pBR322 vector. After transformation into competent E. coli HB101 cells, DNA mini-preparations were made from 16 plasmid-containing colonies. The plasmid DNAs were digested using the unique HindIII site within the plasmid. Subsequent digestion with BamHI indicated those plasmids which had apparently regenerated a left BamHI site (see Figure 3.5). One of the 16 Sau3AI subclones (subclone K in the figure) carrying the regenerated BamHI site, contained ≃6.5 Kb of contiguous DNA (see Figure 3.6), and was selected for amplification and purification in E. coli.

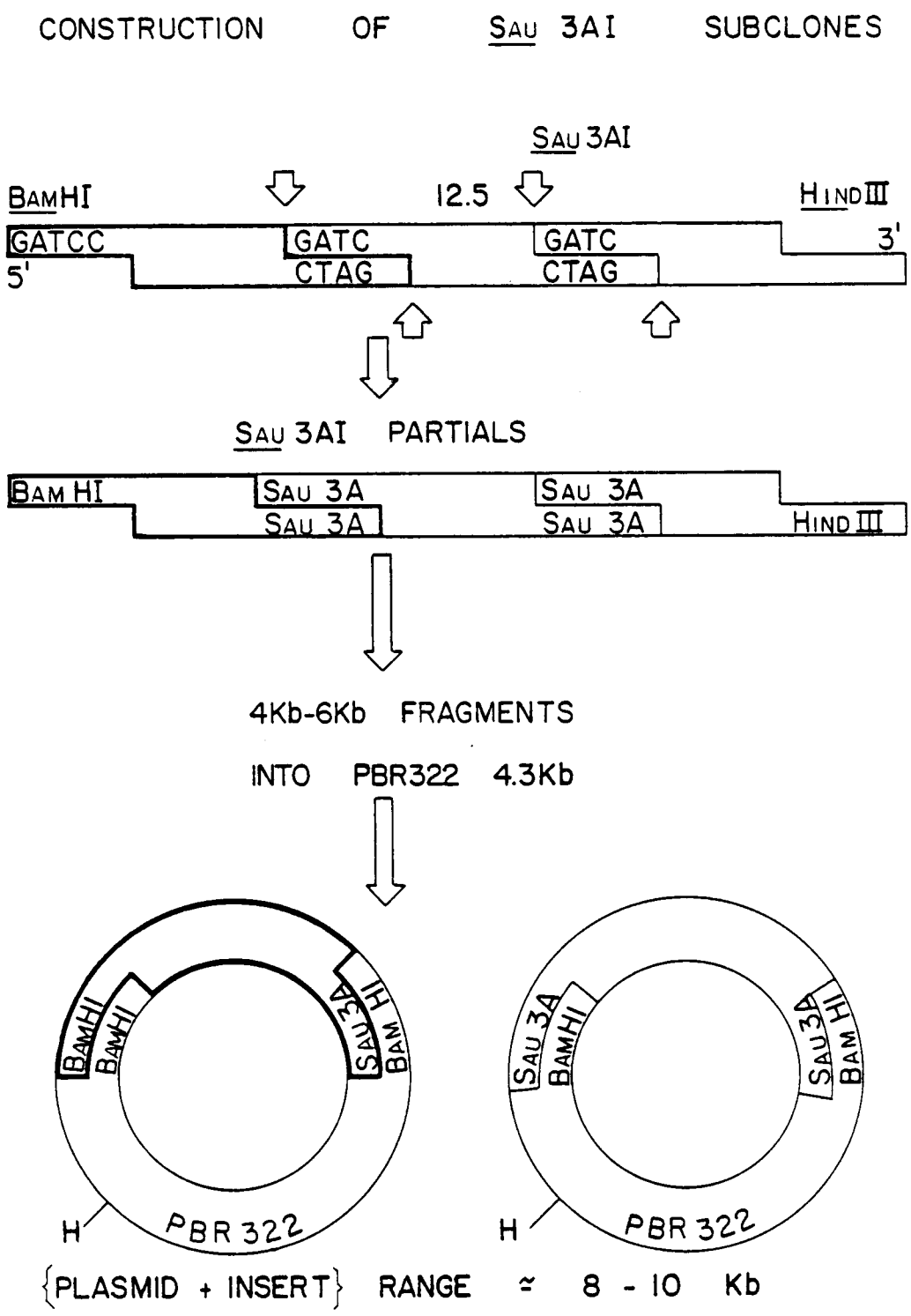
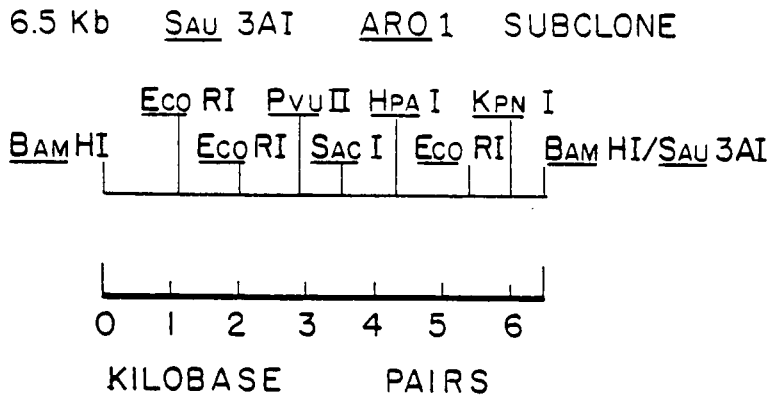


Fig. 3.5. Construction of the Sau3AI subclones (see Figure 3.4, page 34).

left-most BamHI site would be linearized by restriction digests using BamHI. Of the 16 subclones shown in Figure 3.4, only three were digested with BamHI (data not shown). One of these, the subclone containing 6.5 Kb of DNA was chosen for restriction enzyme analysis and complementation studies in the available E. coli, aro mutants. The results of these studies are shown in Figure 3.6. The 6.5 Kb ARO1 subclone complemented all of the available aro mutants of E. coli, which strongly suggested that this 6.5 Kb fragment of DNA contained all of the structural information for expression of the ARO1 gene. The plasmid DNA purified from this subclone was used in the subcloning experiments which follow.

Subcloning of the 6.5 Kb ARO1 Fragment

In an effort to localize the ARO1 gene on the smallest possible segment, the 6.5 Kb ARO1 fragment was subcloned into seven distinct fragments of increasingly larger sizes from the common left-most BamHI site on the 6.5 Kb fragment shown in Figure 3.7. The inserts for all of the seven subclones were obtained from either partial or complete digests of the appropriate DNAs, and subsequently purified by electroelution from 0.7 agarose gels. Where necessary, BamHI linkers were used to construct BamHI termini in order to facilitate pBR322 BamHI site subcloning and subsequent subcloning studies using the BamHI site in the YEp13 shuttle-vector for later use in yeast complementation studies. All purified inserts were ligated into either the BamHI site of pBR322 or into pBR322 missing the



6.5 Kb FRAGMENT
E. coli

COMPLEMENTATION + = COMPLEMENTATION
RESULTS - = NO COMPLEMENTATION

SK 494 (aro E)	+
SK 890 (aro B)	+
SK 2881 (aro D6)	+
SK 3337 (aro A2)	+

Fig. 3.6. E. coli complementation results using the 6.5 Kb fragment. The 6.5 Kb ARO1 fragment was subclone K shown in Figure 3.4. Competent aro strains of E. coli were transformed with 1-2 µg of purified 6.5 Kb/pBR322 plasmid DNA. The transformation mixture was plated directly onto selective agar medium which lacked the aromatic amino acid supplements. Transformants were "cured" of plasmid/insert DNA to insure that the aro phenotype was due to the transforming DNA. As shown in the figure, all aro strains of E. coli were complemented after transformation with the 6.5 Kb/pBR322 plasmid DNA. Complementation: "+" means the ability of a particular aro mutant after transformation, to grow vigorously on nonsupplemented minimal medium (comparable to growth of an aro mutant on supplemented minimal medium). Complementation: "-" means no growth was observed. Those restriction endonuclease sites used in subsequent subcloning experiments are also shown in the figure.

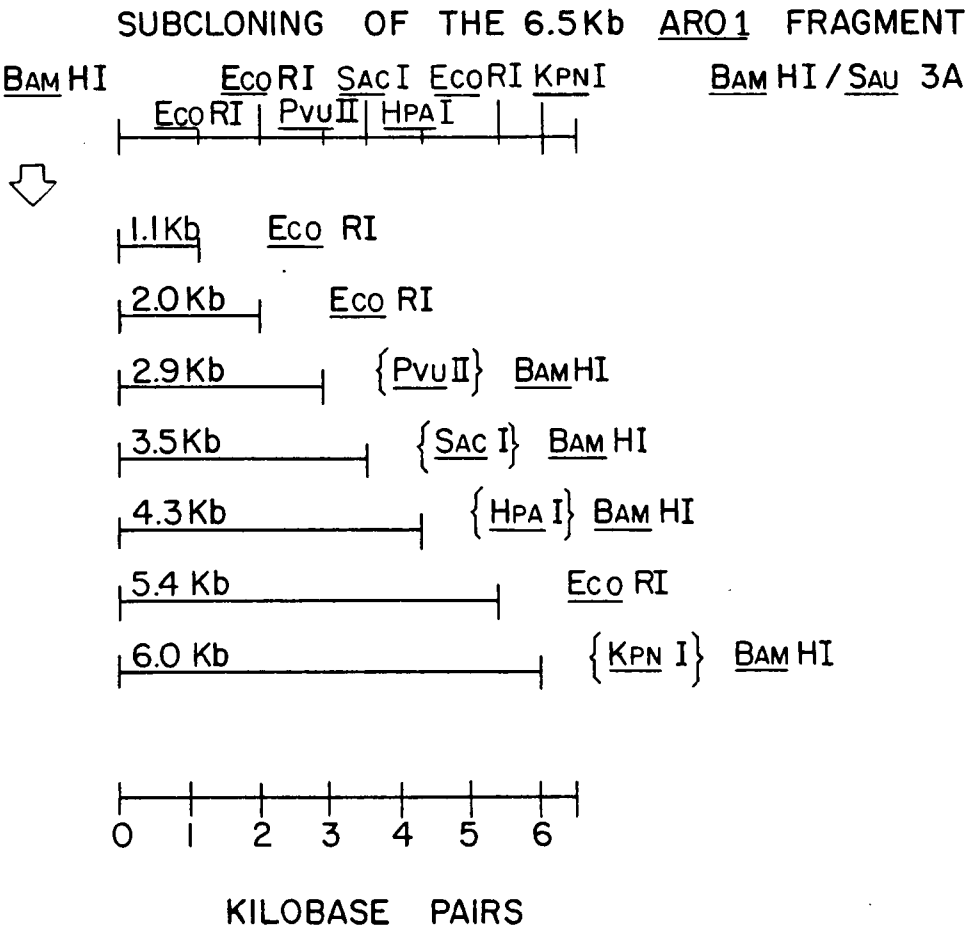


Fig. 3.7. Scheme for subcloning of the 6.5 Kb ARO1 fragment (see Figures 3.8-3.14).

BamHI-EcoRI segment as shown in the constructions (see Figures 3.8-3.14).

Schematic diagrams for the constructions of the 6.5 Kb ARO1 fragment subclones, the 1.1 Kb, 2.0 Kb, 2.9 Kb, 3.5 Kb, 4.3 Kb, 5.4 Kb and the 6.0 Kb are shown in Figures 3.8-3.14. These seven subclones were used in complementation studies using aro mutants of yeast and of E. coli.

Yeast and E. coli Complementation Experiments Using the 6.5 Kb ARO1 Subclones

Since all of the 6.5 Kb ARO1 subclones (see Figure 3.7) were constructed in the E. coli plasmid, pBR322, complementation assays with aro mutants of E. coli were done without further modifications of the constructions. However, each subclone to be tested for its ability to complement aro1 mutants of yeast, required that the insert first be removed from the pBR322 vector and subcloned into YEp13. The 6.0 Kb, 4.3 Kb, 3.5 Kb, and 2.9 Kb BamHI inserts were electroeluted from 0.7% agarose gels. These inserts were re-ligated into the BamHI digested YEp13 vector that had been treated with alkaline phosphatase. The ligation mixture was used in a transformation with competent E. coli HB101 cells. Restriction analysis of DNA mini-preparations of insert-containing colonies confirmed the existence of the appropriate fragment within the YEp13 vector. Aliquots of the appropriate DNA mini-preparations were used in subsequent transformation and complementation assays

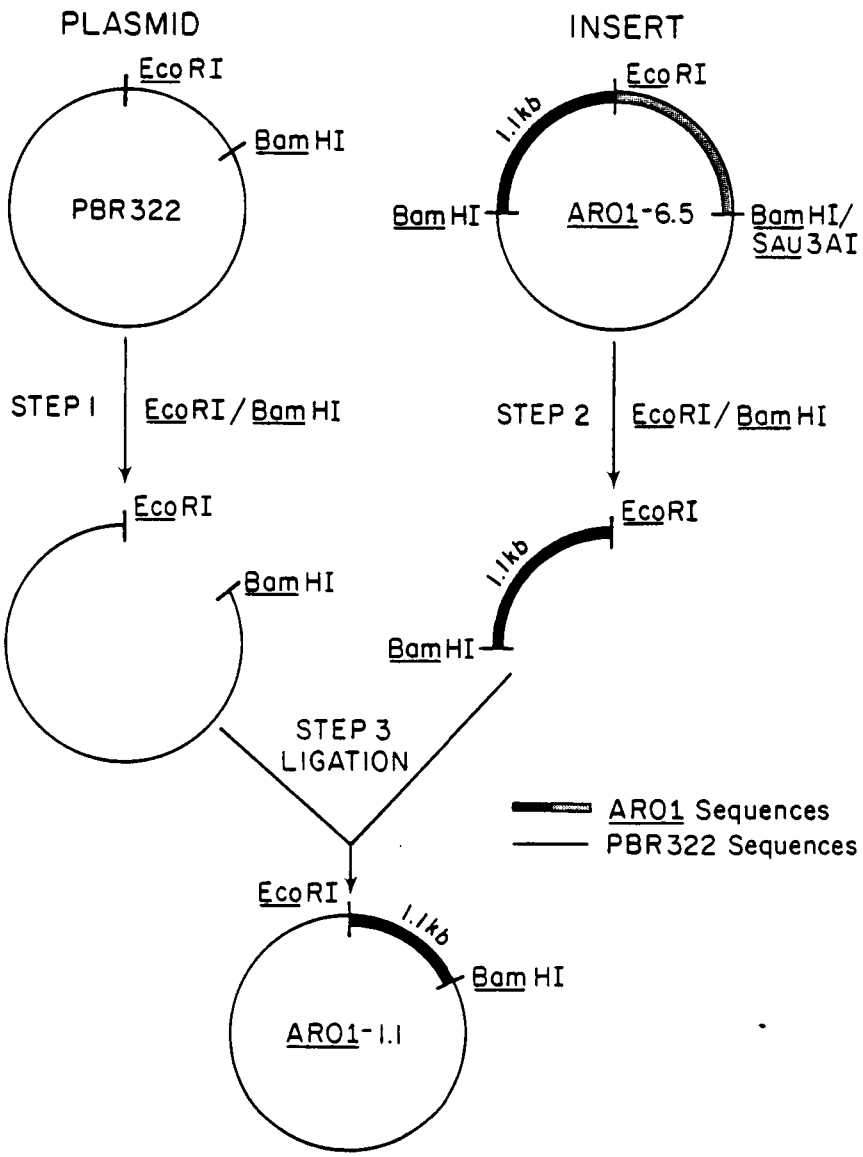


Fig. 3.8. Construction of the ARO1-1.1 subclone. The ARO1-1.1 subclone was constructed from pBR322 and the ARO1-6.5 subclone (see Figure 3.7). The "plasmid" DNA was generated from an EcoRI/BamHI double-digest of pBR322 (step 1). The ARO1-1.1 "insert" DNA was generated from an EcoRI/BamHI double-digest of the ARO1-6.5 DNA subclone (step 2). The resultant plasmid and insert fragments were electroeluted from 0.7% agarose gels as described and ligated together using T4 DNA ligase (step 3). Subsequent transformation into competent E. coli HB101 cells, DNA mini-preparations, and restriction analysis confirmed the construction.

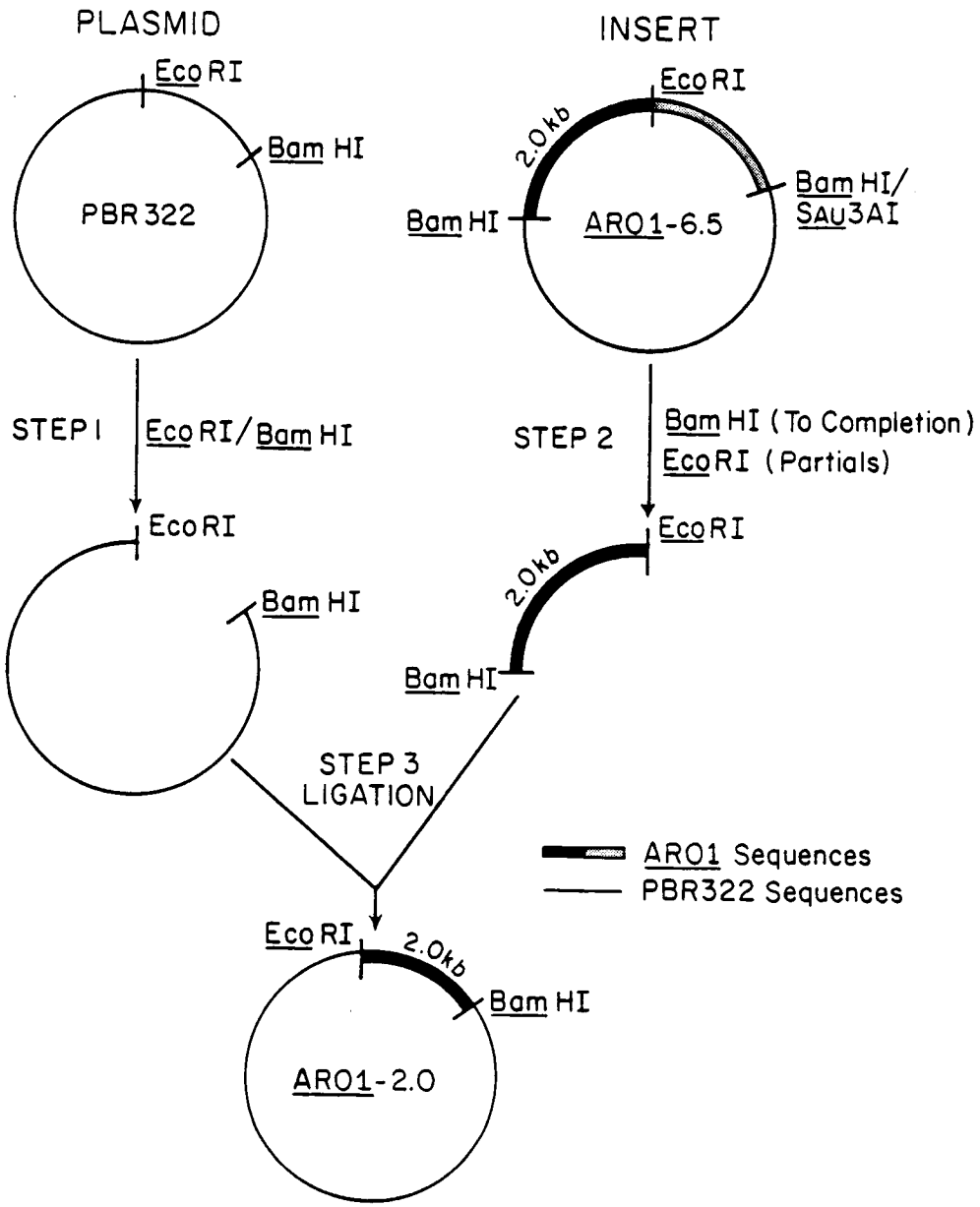


Fig. 3.9. Construction of the ARO1-2.0 subclone. The ARO1-2.0 subclone was constructed from pBR322 and the ARO1-6.5 subclone. The "plasmid" DNA was generated from an EcoRI/BamHI double digest of pBR322 (step 1). The "insert" DNA was generated after digestion of ARO1-6.5 DNA with BamHI to completion followed by partial digestion with EcoRI (step 2). The plasmid and insert fragments were electro-eluted from 0.7% agarose gels as described, and ligated together using T4 DNA ligase (step 3). Subsequent transformation into competent E. coli HB101 cells, DNA mini-preparations, and restriction analysis confirmed the construction.

ARO1-2.9 SUBCLONE

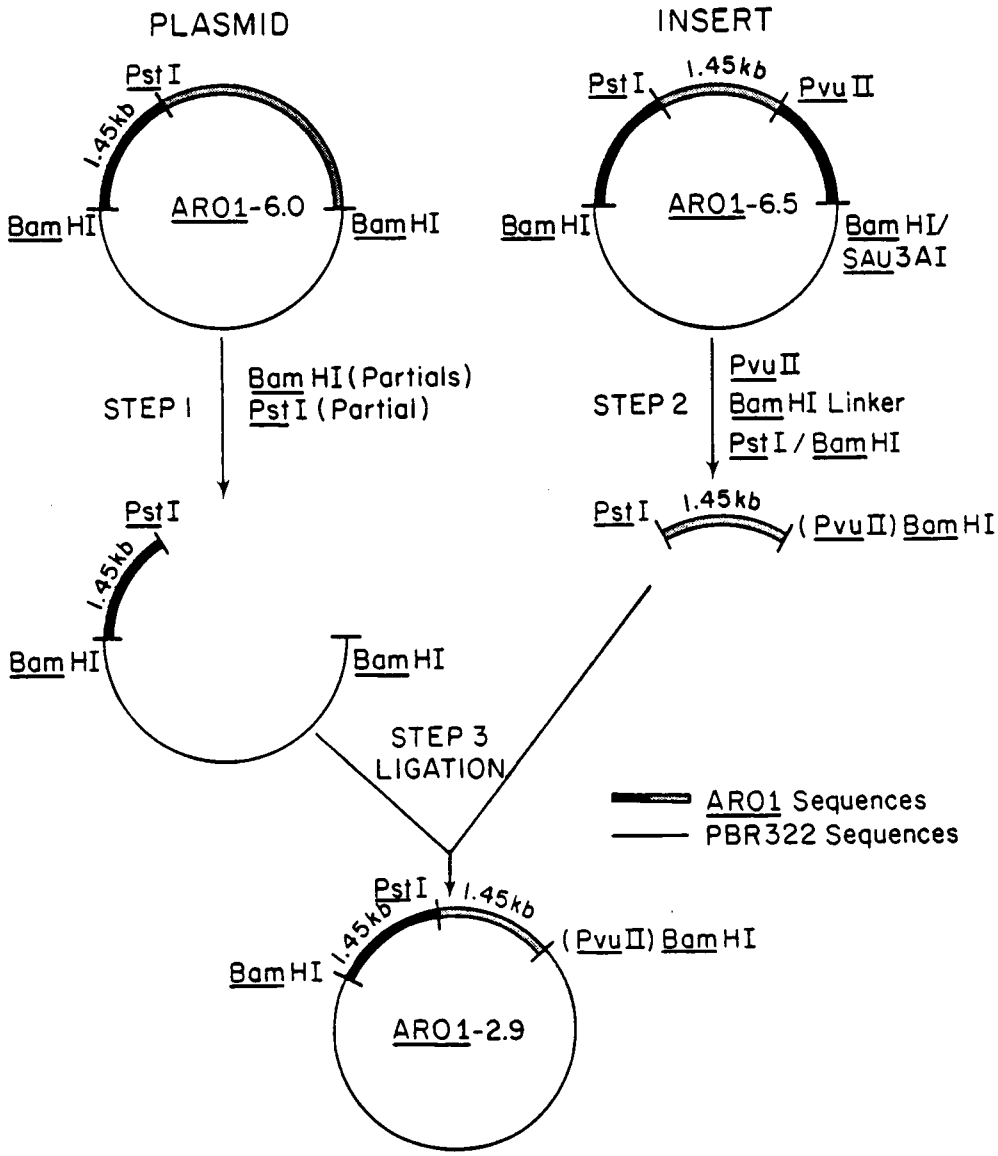


Fig. 3.10. Construction of the ARO1-2.9 subclone. The ARO1-2.9 subclone was constructed from the ARO1-6.0 and the ARO1-6.5 subclones. The "plasmid" DNA was generated from BamHI and PstI partial digests of ARO1-6.0 DNA (step 1). The "insert" DNA was generated after digestion of ARO1-6.5 DNA with PvuII, addition of BamHI linkers, and a subsequent PstI/BamHI double digestion (step 2). The plasmid and insert fragments were electroeluted from 0.7% agarose gels as described, and ligated together using T4 DNA ligase (step 3). Subsequent transformation into competent E. coli HB101 cells, DNA minipreparations, and restriction analysis confirmed the construction.

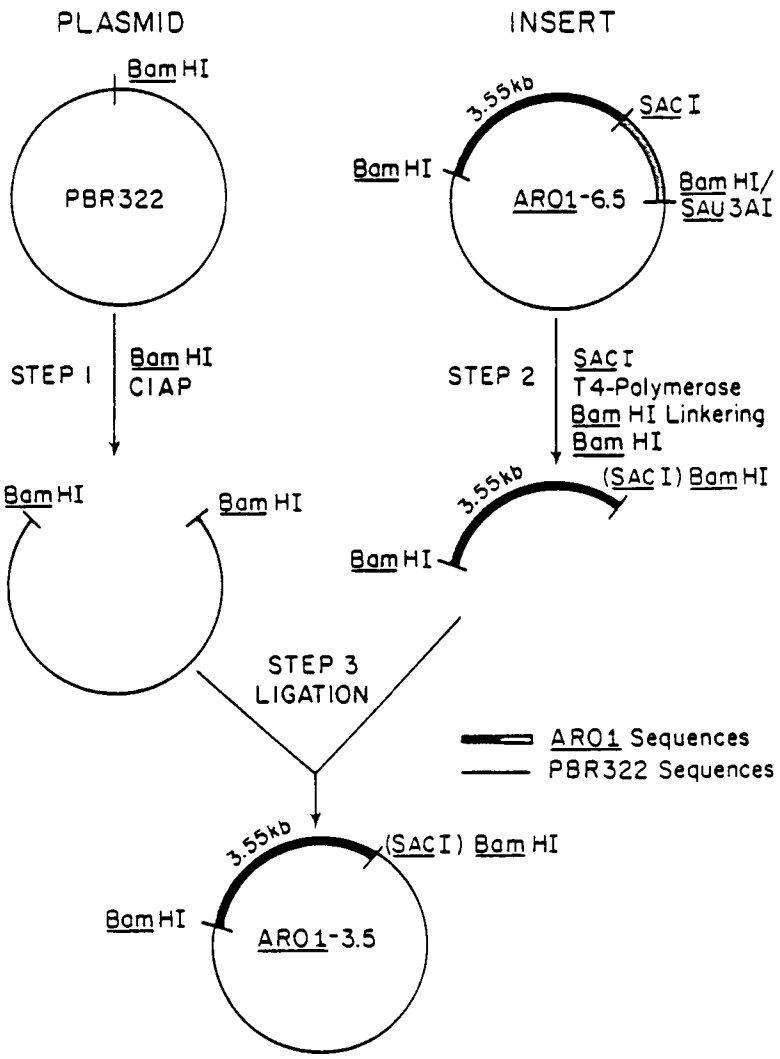


Fig. 3.11. Construction of the ARO1-3.5 subclone. The ARO1-3.5 subclone was constructed from pBR322 and the ARO1-6.5 subclone. The "plasmid" DNA was generated after BamHI digestion of pBR322 (step 1). The "insert" DNA was generated after SacI digestion of the ARO1-6.5 DNA followed by T4 DNA polymerase (3' EXO activity) digestion, addition of BamHI linkers, and a SacI/BamHI double digestion (step 2). The plasmid and insert fragments were electroeluted from 0.7% agarose gels as described, and ligated together using T4 DNA ligase (step 3). Subsequent transformation into competent E. coli HB101 cells, DNA mini-preparations, and restriction analysis confirmed the construction.

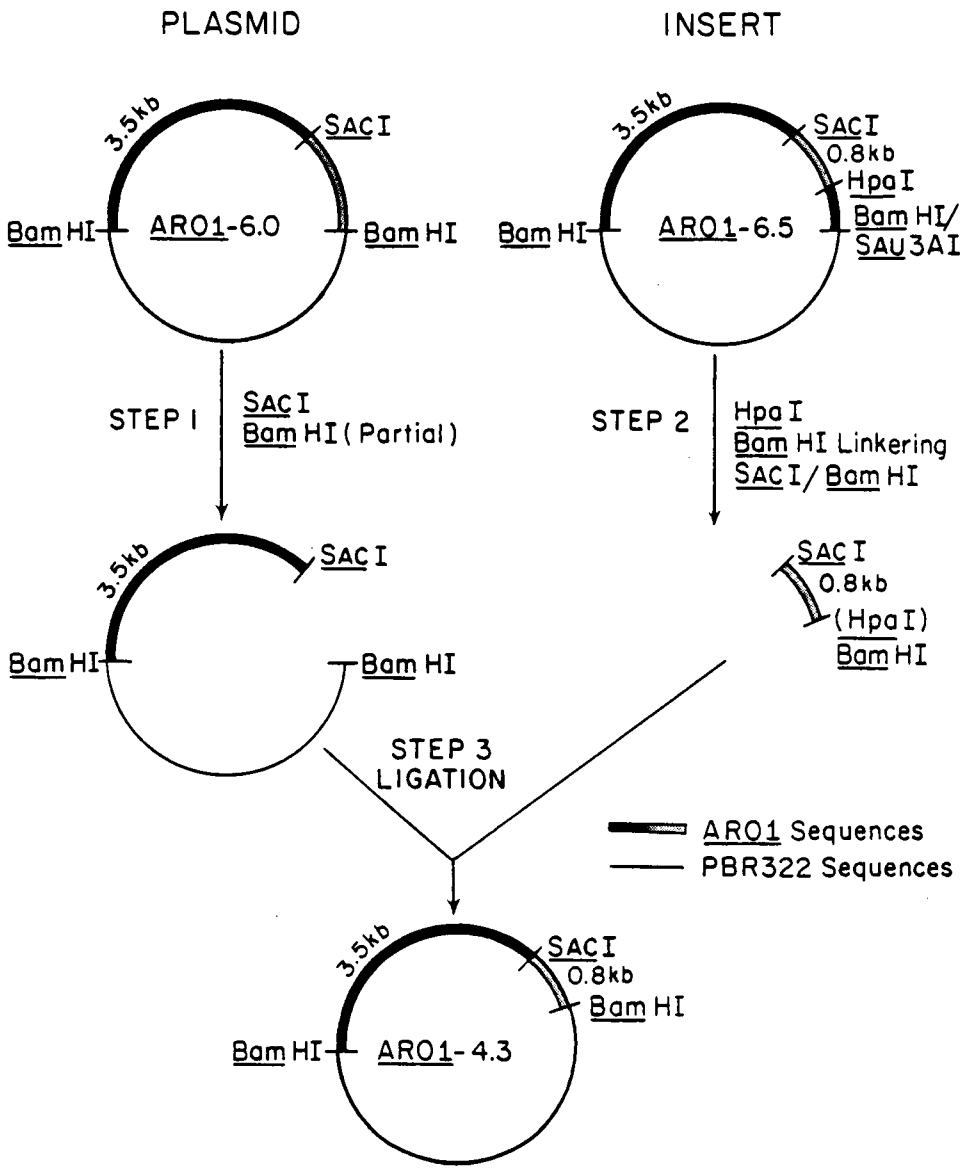


Fig. 3.12. Construction of the ARO1-4.3 subclone. The ARO1-4.3 subclone was constructed from the ARO1-6.0 and the ARO1-6.5 subclones. The "plasmid" was generated with a SacI digestion, followed by BamHI partial digestion (step 1). The "insert" DNA was generated from the ARO1-6.5 DNA after HpaI digestion, addition of BamHI linkers, and a SacI/BamHI double digestion (step 2). The plasmid and insert fragments were electroeluted from 0.7% agarose gels as described, and ligated together using T4 DNA ligase (step 3). Subsequent transformation into competent E. coli HB101 cells, DNA mini-preparations, and restriction analysis confirmed the construction.

ARO1-5.4 SUBCLONE

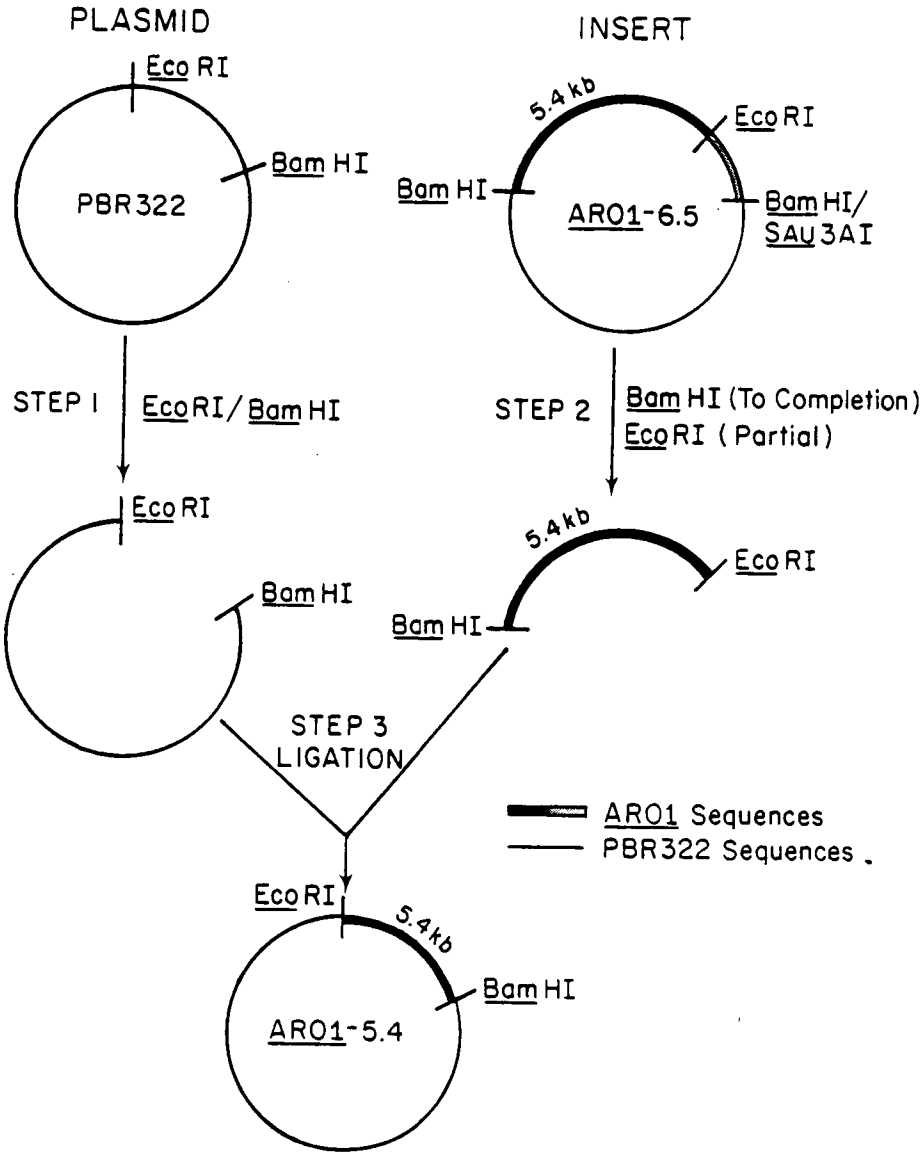


Fig. 3.13. Construction of the ARO1-5.4 subclone. The ARO1-5.4 subclone was constructed from pBR322 and the ARO1-6.5 subclone. The "plasmid" DNA was generated by EcoRI/BamHI double digests of pBR322 (step 1). The "insert" DNA was generated by digestion of ARO1-6.5 DNA with BamHI, followed by an EcoRI partial digestion (step 2). The plasmid and insert fragments were electroeluted from 0.7% agarose gels as described, and ligated together with T4 DNA ligase (step 3). Subsequent transformation into competent E. coli HB101 cells, DNA mini-preparations, and restriction analysis confirmed the construction.

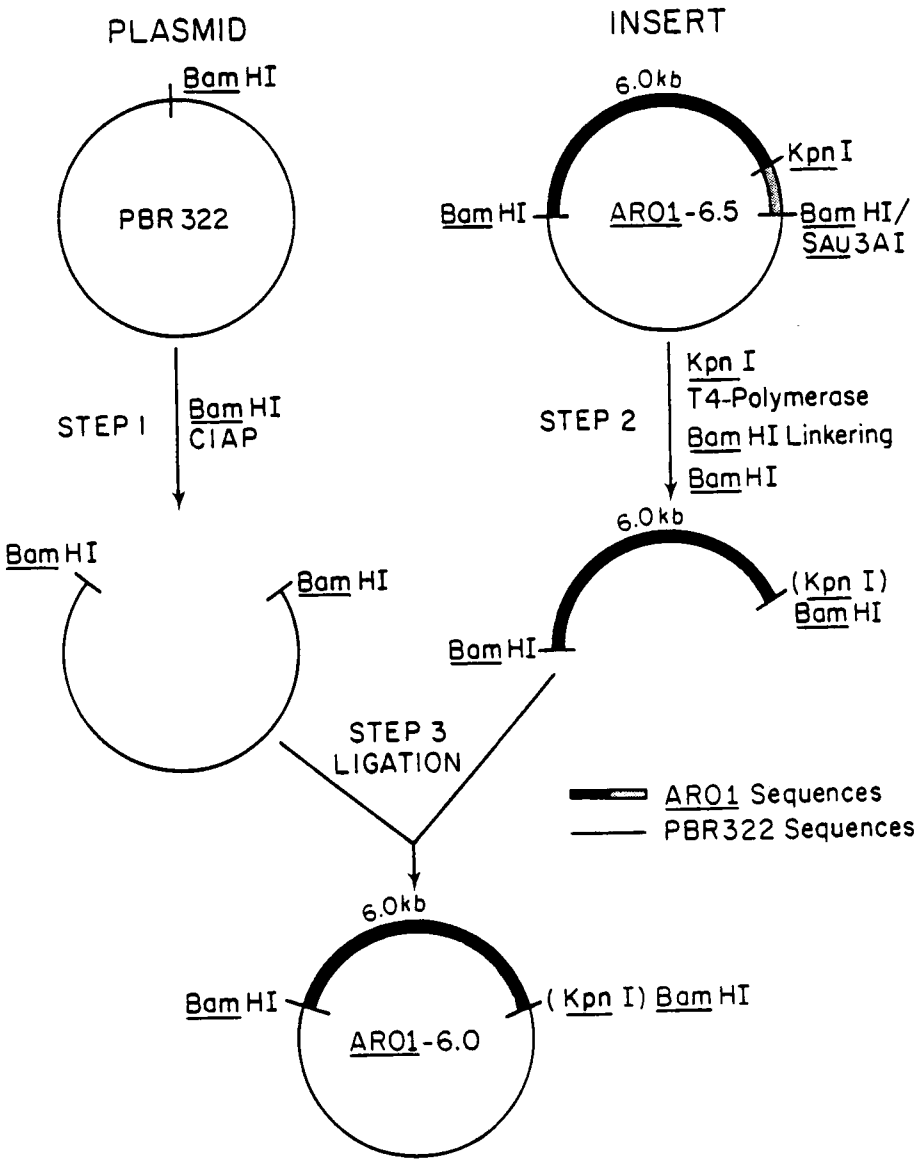


Fig. 3.14. Construction of the ARO1-6.0 subclone. The ARO1-6.0 subclone was constructed from pBR322 and the ARO1-6.5 subclone. The "plasmid" DNA was generated after BamHI digestion of pBR322 (step 1). The "insert" DNA was generated by digestion of ARO1-6.5 DNA with KpnI, followed by T4 DNA polymerase treatment to generate blunt termini. After the addition of BamHI linkers, extensive BamHI digestion generated the 6.0 Kb fragment insert (step 2). The plasmid and insert fragments were electroeluted from 0.7% agarose gels as described, and ligated together with T4 DNA ligase (step 3). Subsequent transformation into competent E. coli HB101 cells, DNA mini-preparations, and restriction analysis confirmed the construction.

in both yeast and in E. coli, aro mutants. The results of these complementation experiments are shown in Figure 3.15. As shown in Figure 3.15, those subclones containing at least 3.5 Kb of contiguous DNA were capable of complementing two aro mutants of yeast (including a pleiotropic mutant) and all of the available aro mutants of E. coli. Interestingly, the 2.9 Kb subclone was capable of complementing only one aro mutant of E. coli--SK494 (aroE).

RNA/DNA Hybridization-Dot Blot Analysis

A schematic of the RNA/DNA hybridization studies is shown in Figure 3.16. Total RNA was puried from wild type, log phase, yeast (S288C) cells, spotted onto nitrocellulose and probed with a [^{32}P]-5'-labelled EcoRI fragment. The EcoRI fragment probe was chosen because of the internal location of the fragment within the 3.5 Kb ARO1 subclone. After labelling, a PstI digestion generated two distinct, separable, double-stranded fragments, each containing a single label on an opposite strand. These fragments, a 350(N) and a 550(N), were electrophoresed in and purified from 5% polyacrylamide gels. Each fragment was used in independent hybridization studies to probe two separate RNA preparations. The 550(N) EcoRI probe hybridized to both RNA preparations (see Figure 3.17), whereas the 350(N) EcoRI probe failed to hybridize with either preparation (data not shown).

	Kb						
	1.1	2.0	2.9	3.5	4.3	5.4	6.0
Yeast							
XL39-4A (aro1D nonsense)	NT	NT	--	+	+	NT	+
XL60-2B (aro1D nonsense)				+	+	NT	+
XL61-1D (aro1E missense)	NT	NT	--	+	+	NT	+
E. coli							
SK494 (aroE)	--	--	+	+	+	+	+
SK890 (aroB)	--	--	--	+	+	+	+
SK2881 (aro1D)	--	--	--	+	+	+	+
SK3337 (aroA2)	--	--	--	+	+	+	+

+ = Complementation.
- = No complementation.
NT = Not tested

Fig. 3.15. Yeast and E. coli complementation results using the ARO1-6.5 Kb subclones. Seven subclones of increasingly larger sizes from a common BamHI end (see Figures 3.7-3.14) were constructed. Aliquots of the appropriate DNAs (1-10 µg) were used in transformation and complementation assays using competent strains of yeast or of E. coli. Complementation: "+" means the ability of a particular aro mutant, after transformation, to grow vigorously on nonsupplemented minimal medium (comparable to growth of an aro mutant on supplemented minimal medium). Complementation: "-" means no growth was observed.

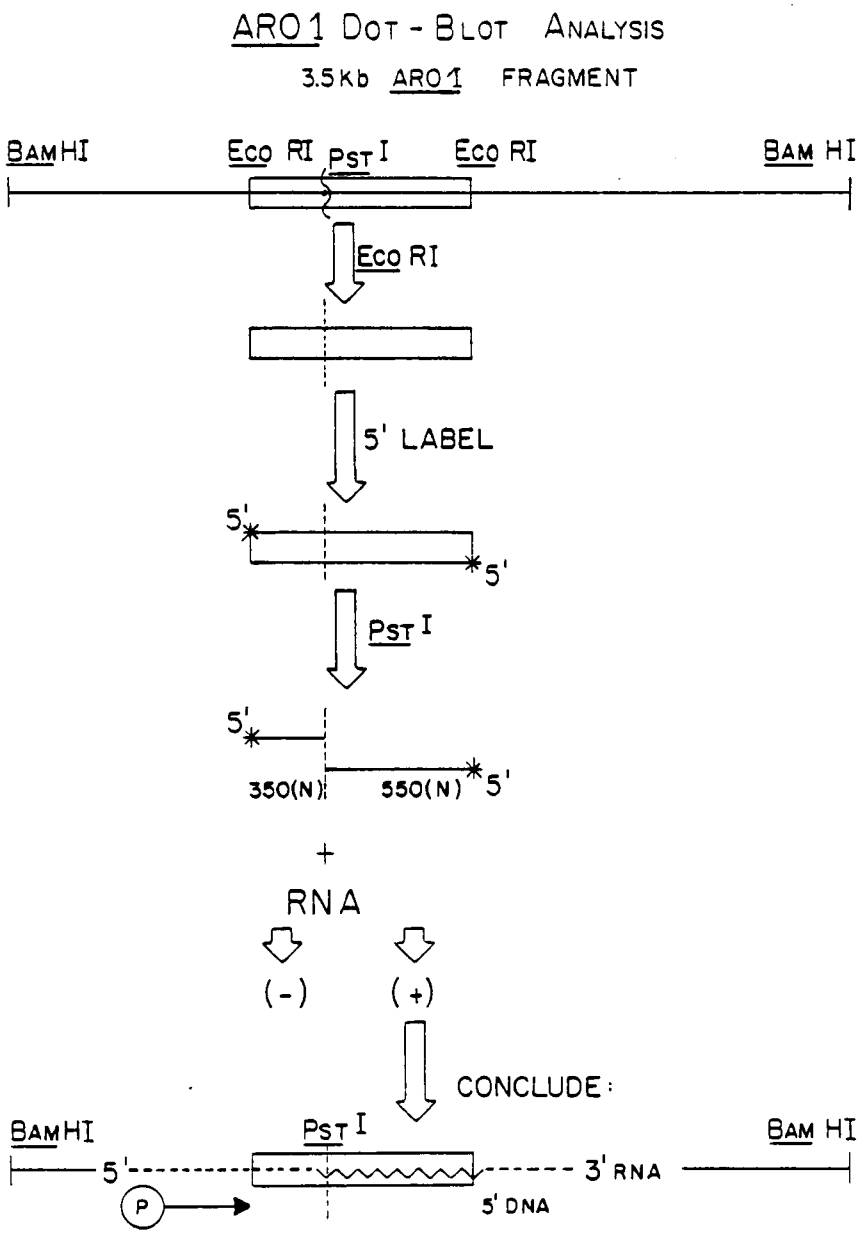


Fig. 3.16. Schematic for dot-blot analysis. The DNA segment shown at the top of the figure is the 3.5 Kb fragment (see Figure 3.7). An internal EcoRI segment (≃1 Kb) was purified from the 3.5 Kb fragment, [^{32}P]-5'-labelled, and digested with PstI. The resulting single-end labelled fragments (350 (N) and 550 (N)) were used to probe total RNA purified from wild type yeast (S288C) cells. "+" means the DNA probe hybridized to the RNA. "-" means the DNA probe failed to hybridize to the RNA (see Figure 3.17).

RNA #1 RNA #2

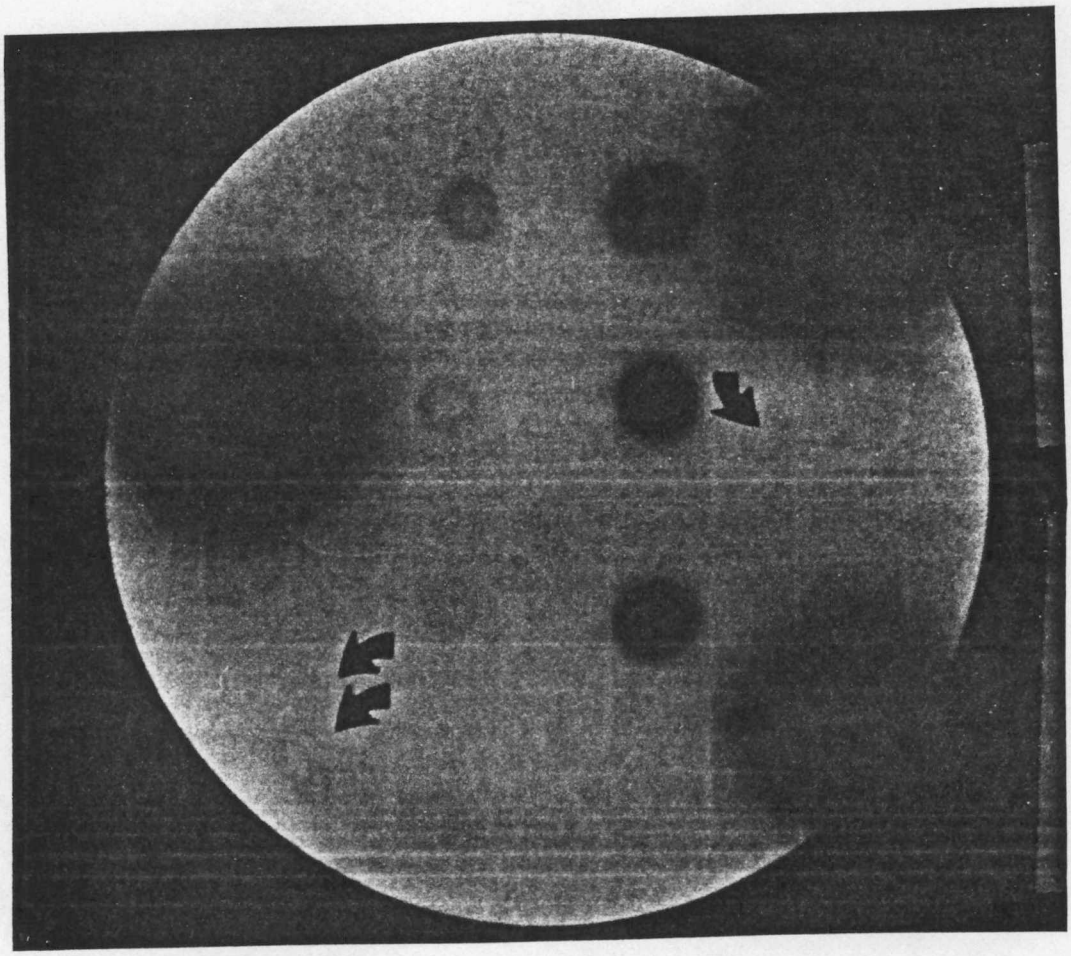


Fig. 3.17. RNA/DNA hybridization-dot blot analysis. The two columns of RNA represent total RNA preparations from wild-type yeast, S288C cells. The RNA was serially diluted and spotted onto nitrocellulose; the most concentrated spot is represented by the top-most dot. Positive control DNA (∿10 ng) was spotted in three different positions on the filter (represented by the three large, over-exposed regions). Negative control areas are indicated by the single arrow and the double arrow: the single arrow was a commercial preparation of yeast t-RNA, and the double arrow was genomic RNA purified from the mushroom virus (a generous gift from Leon Stankowski). The filter was then probed with a [^{32}P]-5'-labelled 350 (N) or 550 (N) EcoRI fragment. The 350 (N) fragment failed to hybridize to either RNA preparation (data not shown); only the 550 (N) fragment hybridized to the wild-type RNA preparations.

Nucleotide Sequences

The DNA nucleotide sequencing scheme is shown in Figure 3.18. The restriction sites shown were used to generate fragments which were [^{32}P]-5'-labelled and sequenced according to the Maxam-Gilbert protocol (38, 39); see Figure A.3, page 70. Partial sequence information has been determined for three distinct regions within the 2 Kb segment of the ARO1-2.0 Kb subclone (see Figure 3.9, page 41). The completed sequences are represented as darkened "blocked" areas in Figure 3.18 and are shown in Figures 3.19, 3.20, and 3.21. The most important preliminary find came from within a sequenced region of approximately 400 nucleotides starting at the left-most BamHI site. These sequences demonstrated the existence of a potential yeast type TATA promoter region (18) (see Figure 3.19).

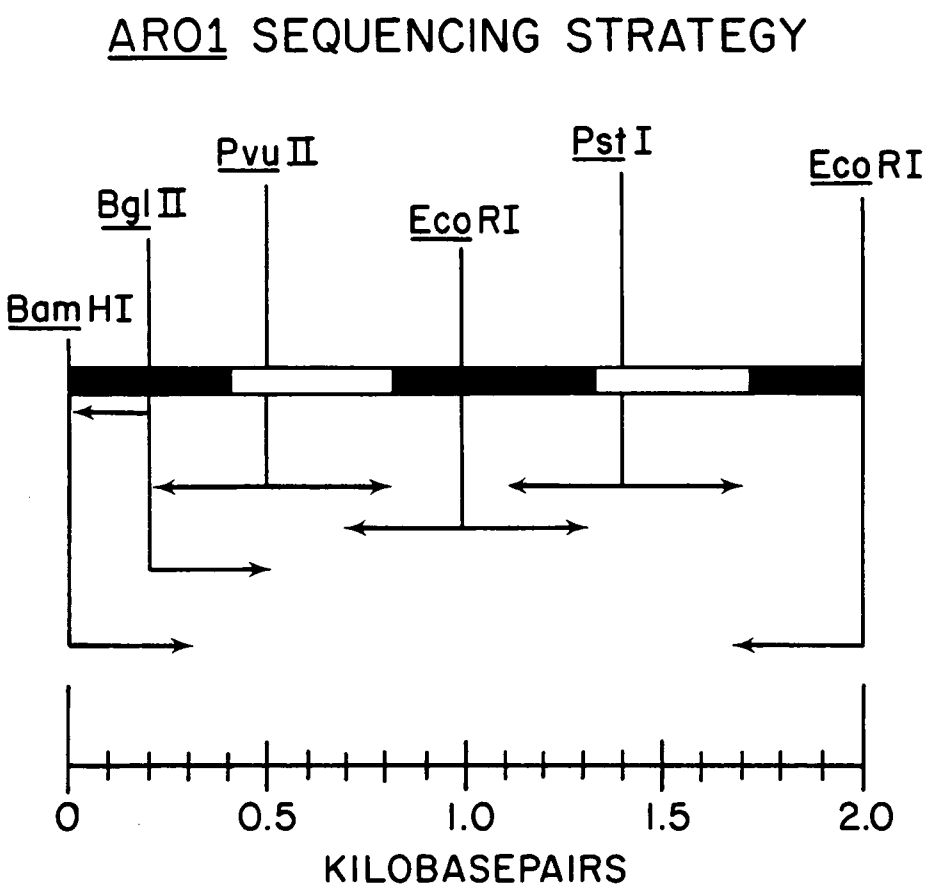


Fig. 3.18. ARO1 sequencing strategy. Partial sequence information has been determined for three distinct regions within a 2 Kb segment of the ARO1-2.0 Kb subclone (see Figure 3.9, page 41). The completed sequences are represented as the dark blocked areas in the figure. The restriction sites shown were used to generate fragments which were [^{32}P]-5'-labelled and sequenced according to the Maxam-Gilbert protocol.

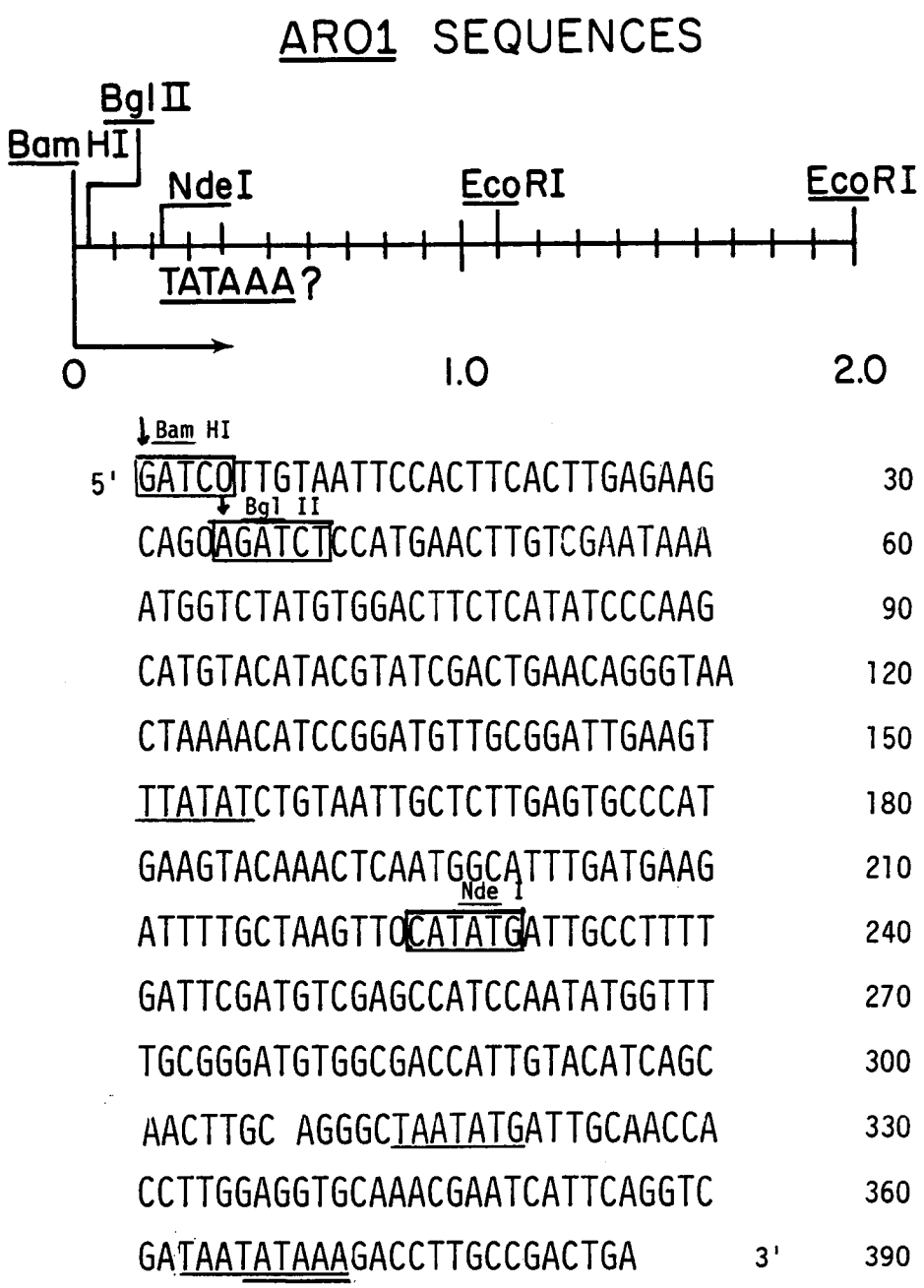


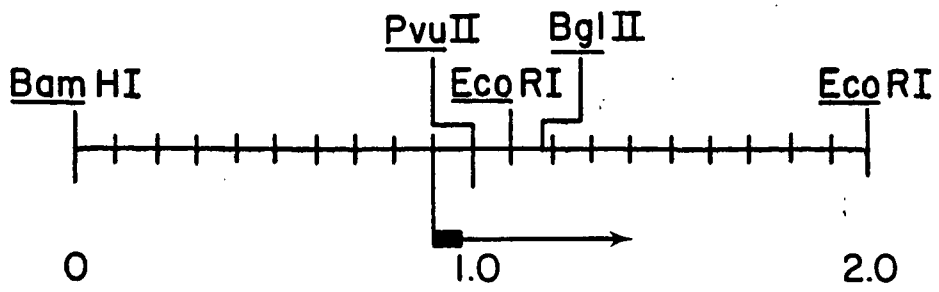
Fig. 3.19. ARO1 sequences (A). DNA sequences from the left-most region of the ARO1-2.0 subclone (see arrow above and Figure 3.9). Blocked areas represent restriction endonuclease sites and underlined sequences represent possible promoter regions. The double-underlined sequence represents a typical yeast type "TATA" promoter.

ARO1 SEQUENCES


```
5'  CCAATTAAGGTTGGTACAAAAGCAGCATGG                    30
    GCACCTTTCACAAGGAATCTCTCCAGCTTA                    60
    CTTAAAAGTTCGTCGTCTGGTGGTTTGTCG                    90
    GCTGGTACACTGCAGACAGCAACGCCAACG                   120
              PvuII
    TGTTCCTTGGTCTCTTCTGTTGATTTAGTA                   150
    GACTCTATTCCAATGGTGTTGAATTCAGCA                   180
                 EcoRI
    GACGGAAGTGATCTCACCAATGGCCCCAAT                   210
    TTCGAAGTTGTCCTGTTACCACGAAGATCT                   240
                 BglII
    TTTTGCAACCTAAACTGTGTAAGGTGTAAA                   270
    GGGTGGTT                                3'       300
```

Fig. 3.20. ARO1 sequences (B). DNA sequences from the middle region of the ARO1-2.0 subclone (see arrow above and Figure 3.9). Blocked areas represent restriction endonuclease sites. Vertical lines represent the three different reading frames.

ARO1 SEQUENCES




```
5'  CCA GAGTATAATCCTTGGACCATGATGGG          30

    AACCAAGGTCTTGGTGTTGCCCCTTTTGTT          60

    AATAACCTCATATTGGATATCAGTAGGTAA          90

    AGTTAGTTCTAAGTCAAGGAATT      3'        120
```

Fig. 3.21. ARO1 sequences (C). DNA sequences from the rightmost region of the ARO1-2.0 subclone (see arrow above and Figure 3.9, page 41). Vertical lines represent the reading frames.

CHAPTER IV

DISCUSSION AND SUMMARY

The studies presented in this thesis are essentially a continuation of the ARO1 subcloning studies initiated by Larimer et al. (31). The original fragment containing the ARO1 gene was a 17.2 Kb BamHI fragment isolated by complementation in Saccharomyces cerevisiae following transformation with a comprehensive yeast DNA library of BamHI restriction fragments inserted into the shuttle-vector YEp13 (10, 27, 31; see Figure A.2, page 69). The present study used recombinant DNA technology (36, 29) to isolate and characterize the ARO1 gene of Saccharomyces cerevisiae. The ARO1 gene is thought to be a "cluster-gene"--a single transcriptional unit encoding a pentafunctional polypeptide. The ARO1 gene product has an estimated molecular weight of approximately 150,000 daltons (31) and catalyzes reactions 2 through 6 in the biosynthesis of the aromatic amino acids (see Figure 1.1, page 4). The "cluster-gene" model proposed for the ARO1 gene stemmed from earlier work on the "arom aggregates" of other fungal systems (22, 24, 13, 12, 23); however, the definite studies have not been done.

Because of the relative ease with which E. coli cells can be transformed, it was of interest to determine if the original 17.2 Kb ARO1 fragment (see Figure 3.1, page 28) was capable of being expressed in E. coli hosts. The expression of the 17.2 Kb BamHI fragment in two different orientations relative to plasmid (see

Figure 3.2, page 30) in four different aro mutants of E. coli demonstrated not only the ability of the yeast ARO1 gene to be expressed by E. coli, but also that the expression, in E. coli, was due to a yeast promoter on the fragment, presumably due to the ARO1 promoter, and not due to expression off the tetracycline promoter on the plasmid. It should also be pointed out that the expression of the ARO1 gene in E. coli strongly suggests the lack of introns within the ARO1 gene. However, the expression results do not preclude the possibility that small intronic regions are present that are fortuitously transcribed and translated without affecting complementing activity in E. coli.

Complementation assays, using subcloned fragments inserted into either pBR322 (9, 48), or YEp13 (10, 27) were performed in nonsense and missense mutants of ARO1 in yeast and in four separate aro mutants of E. coli (see Figure 3.15, page 48). The figure demonstrates that the 2.9 Kb subclone (see Figure 3.10, page 42) failed to complement aro mutants of yeast, but does demonstrate complementing activity in one of the available aro mutants of E. coli (see Figure 3.15, page 48). The removal of 600 nucleotides from the 3.5 Kb subclone (to construct the 2.9 Kb subclone) may have removed critical polyadenylation sites necessary for messenger RNA maturation and translation; and, therefore, may explain the expression in E. coli and the lack of expression in aro mutants of yeast. It is not surprising that complete ARO1 gene activity is not found in aro mutants of E. coli transformed with the 2.9 Kb fragment, since the 2.9 Kb

subclone theoretically contains less genetic information than is required for expression of a complete ARO1 gene system. However, the complementing activity of the 2.9 Kb subclone in only one of aro mutants of E. coli indicates that at least a fragmented protein, with partial activity, is being formed. The existence of only one observable complementary activity out of the four aro mutants of E. coli transformed with the 2.9 Kb ARO1 subclone may be due to the inability of the fragmented protein to form into the crucial tertiary structures required by the different domains within the primary structure of the ARO1 gene product. The complementation results shown in the figure also suggest that a functional ARO1 gene is located within the 3.5 Kb subclone. Based on the estimated 150,000 dalton molecular weight of the protein product (31), the minimum size of a DNA fragment capable of encoding the ARO1 gene is approximately 3.5 Kb to 4.0 Kb; thus, the size of subcloned 3.5 Kb fragment, taking into consideration error(s) involved in either DNA fragment size estimations from agarose gels or in protein size determinations from denaturing polyacrylamide gels, must be very close to the ARO1 gene itself.

RNA/DNA hybridization studies (see dot-blot analysis, page 47) were performed in an effort to distinguish between "sense" and "anti-sense" strands for the ARO1 gene on the 3.5 Kb fragment. RNA purified from log phase cells of S288C was attached to nitrocellulose and probed with a [^{32}P]-5'-labelled EcoRI fragment (see Figure 3.16, page 49). The EcoRI fragment was chosen because it occupied the

approximate middle portion of the subcloned 3.5 Kb ARO1 fragment; and therefore should be completely within the ARO1 gene coding sequences. Knowing the chemical polarity of the DNA probe, it was possible to deduce the direction of transcription. The results, shown in Figures 3.16 and 3.17, pages 49 and 50, indicate that the ARO1 gene is transcribed from left to right relative to the fragment orientation shown in Figure 3.7, page 38. These data are consistent with the preliminary DNA sequence. From within a sequenced region proximal to the left BamHI site (see Figure 3.19, page 53), there is a potential yeast type "TATA" promoter (18); no other completed regions of nucleotide sequence information showed any "TATA" regions (see Figure 3.20 and Figure 3.21, pages 54 and 55). The sequences shown in Figures 3.20 and 3.21 come from the middle portion of the the 3.5 Kb ARO1 subclone; and therefore should be completely within the ARO1 coding region. Both of the sequences have only one open reading frame (reading frame #1).

The RNA/DNA hybridization studies and the sequence data in combination with the yeast and E. coli complementation studies (the Bgl II 7.7 Kb subclone, which lacks ≈1 Kb of left-most nucleotide sequence information (see Figure 3.3, page 32) fails to complement aro mutants of either yeast or of E. coli) demonstrates the apparent production of a non-spliced ARO1 transcriptional unit initiated near the left-most end of the 3.5 Kb fragment. These data were consistent with the cluster-gene model proposed for the ARO1 gene of Saccharomyces cerevisiae (31), and indicate a possible similarity to

the Saccharomyces cerevisiae HIS4 cluster-gene organization reported by Donahue et al. (19). However, further molecular studies, including S_1-mapping (46, 4) and complete nucleotide sequence data will be necessary in order to conclusively establish the single-transcript, single-polypeptide model proposed for this ARO1 gene system (Larimer and Gaertner, manuscript in preparation).

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APPENDIX

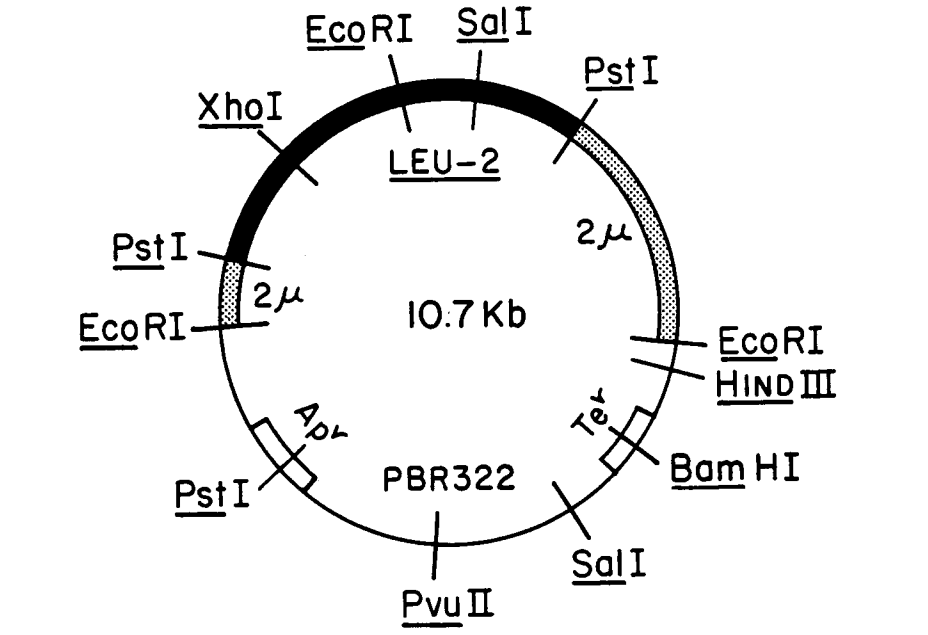

Fig. A.1. Partial organization of the YEp13 hybrid shuttle-vector. The pBR322 sequences in this vector extend from the EcoRI sites which border the 2 micron circle sequences, and include the plasmid origin of replication (when in E. coli) and the ampicillin (Ap^r) and tetracycline (Te^r) resistance regions. The 2 micron circle DNA sequences represent two distinct and unequal fragments containing PstI and EcoRI termini, and contain the plasmid origin of replication required by the yeast replication system. The LEU2 sequences serve as marker sequences when the vector is utilized in a yeast host (see references 10 and 27).

ISOLATION OF THE 17.2 Kb ARO1 FRAGMENT

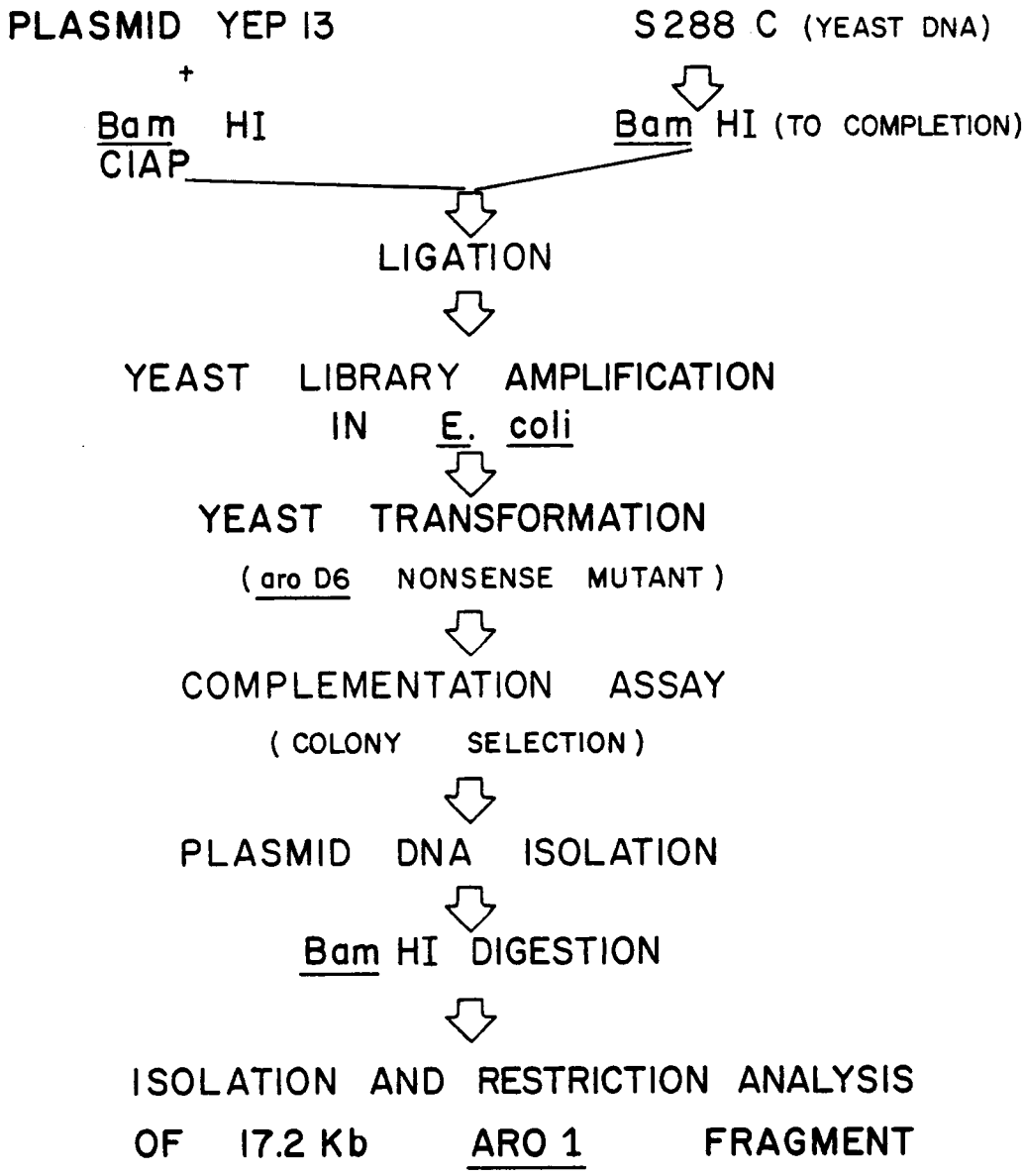

Fig. A.2. Isolation of the 17.2 Kb ARO1 fragment (see reference 31).

DNA SEQUENCING PROTOCOL

DATE: SERIES: FILMS:

GEL BAND:

RESTRICTION LABEL:

	A+G	G	T+C	C
Starting CPMS:				
	10µl ^{32}P-DNA	10µl ^{32}P-DNA	15µl ^{32}P-DNA	10µl ^{32}P-DNA
	10µl H_2O	200µl DMS	5µl H_2O	20 µl (5M) Nacl
	0°C	0°C	0°C	0°C
	2µl Pip.Formate (1 ML Formic Acid + 24 ML H_2O + 2 drops Piperidine)	1µl DMS	30µl HZ	30µl HZ
	37°C 13'	20°C 2'	20°C 8'	20°C 8'
	Freeze (-80°C 10')	50µl DMS Stop	200µl HZ Stop	200µl HZ Stop
		800µl Ethanol	800µl Ethanol	800µl Ethanol
		-80°C 10'	-80°C 10'	-80°C 10'
		SPIN 10'	SPIN 10'	SPIN 10'
		300µl 0.3M N_aOAL	300µl 0.3M N_aOAL	300µl 0.3M N_aOAL
		800µl E+OH	800µl E+OH	800µl E+OH
		-80°C 10'	-80°C 10'	-80°C 10'
		10' SPIN	10' SPIN	10' SPIN
		REPEAT	REPEAT	REPEAT
		2-80% Washes	2-80% Washes	2-80% Washes
	Lyophilize	Lyophilize	Lyophilize	Lyophilize
	100µl H_2O	100µL H_2O	100µl H_2O	100µl H_2O
	10µl PIP.	10µl PIP	10µl PIP	10µl PIP
	90°C 30'	90°C 30'	90°C 30'	90°C 30'
	Freeze -80°C 10'	Freeze -80°C 10'	Freeze -80°C 10'	Freeze -80°C 10'
	Lyophilize O.N.	Lyophilize O.N.	Lyophilize O.N.	Lyophilize O.N.
	Freeze Dry	Freeze Dry	Freeze Dry	Freeze Dry
	2x Next Day	2x Next Day	2x Next Day	2 x Next Day

Fig. A.3. Maxam-Gilbert sequencing protocol.

VITA

Clarence C. [Buzzy] Morse was born August 26, 1948 in Endicott, New York. He graduated from the Owego Free Academy High School in September 1966. He obtained his Bachelor of Arts degree in Biology from The University of Tennessee, Knoxville, in 1973. He worked for five years at The University of Tennessee Memorial Research Center in Nuclear Medicine and was board certified in Nuclear Medicine in 1976. In September 1978 he entered the Ph. D. program in Biomedical Sciences at The University of Tennessee Oak Ridge Graduate School of Biomedical Sciences located at the Biology Division of the Oak Ridge National Laboratory. On September 8, 1978 he was married to Cynthia Courtney. He received his Ph. D. degree in Biomedical Sciences in August 1983, under the guidance and supervision of his major professor, Dr. Sankar Mitra, and his co-advisor, Dr. Frank Larimer. He subsequently joined the research group of Dr. Susan Astrin at the Institute for Cancer Research at the Fox Chase Medical Center in Philadelphia, Pennsylvania to study the molecular basis of non-viral oncogenesis.