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A rapid system for analyzing histone H4 mutations in the yeast Saccharomyces cerevisiae

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Abstract

Genetically engineered, specific histone H4 mutations have been analyzed in yeast which lack normal H4 protein using the time consuming technique of tetrad dissection. The purpose of the present research project has been to develop a rapid new system for analyzing histone H4 mutations in yeast. The new system has made it possible to isolate quickly cells which are surviving on mutant H4 only. The system will make it possible to analyze more quickly large numbers of H4 mutations. It is termed a plasmid shuttle system because of the use of plasmid vectors for shuttling histone H4 alleles in and out of the yeast cell. The system relies on the availibility of haploid yeast cells which have been deleted for both copies of their histone H4 gene and which are dependent on plasmid encoded wild type H4. The system's effectiveness has been tested by analyzing H4 mutations which have been previously examined by another more time consuming method. These mutations have been defined as encoding functional or nonfunctional H4.

An H4 mutation never examined has also been analyzed using the new system. The mutation consists of 6 amino acid substitutions: arginine is replaced by lysine at position 3, glycine by aspartic acid at position 6 and 42, glycine by serine at position 7 and 41, and alanine by threonine at position 38. Statistical analysis of the data provides evidence that this mutation in the H4 gene does not encode functional H4 protein.

A Rapid System For Analyzing Histone H4 Mutations In The Yeast Saccharomyces Cerevisiae

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INTRODUCTION

Chromatin, the hereditary material of cells, is made up of nucleosomes which are repeating units of DNA and protein. The DNA in the nucleus coils around these proteins (histones) forming the bead-like nucleosomes. There are 4 types of histone proteins which make up the nucleosome core particle of these beads: histones H2A; H2B; H3; and H4. In-depth studies of nucleosome structure have been thoroughly reviewed throughout the past decade (Igo-kemenes et al., 1982; Isenberg, 1979; Lilley and Pardon, 1979; McGhee and Felsenfeld, 1980; Pederson et al., 1986).

Chromatin structure may have roles in a number of cellular processes, including DNA replication and the control of gene expression. Therefore a study of the structure-function relationships of the histones may provide biologically significant information regarding cell function at the molecular level. For example, by making specific mutations in the H4 protein, we can determine parts of the protein molecule which are critical for proper function. We can ask questions such as: Does the cell live with this mutation? If so, what new characteristics are associated with the mutation? Do these new characteristics tell us anything about other cellular interactions in which H4 is involved ? Histone H4 is one of the most highly conserved proteins known throughout evolution which suggests that some crucial structural

element of H4 is required for life itself. In fact, the amino acid sequence of H4 in yeast strongly resembles that found in the cow having only 8 differences out of 102 amino acids (Stephenson, 1984). Genetically engineered, specific H4 mutations have been analyzed in yeast which lack any other source of H4 protein. The purpose of the present investigation has been to develop a rapid new system for mutational analysis and to use the system for examining one H4 mutation.

The current structural model of the nucleosomal core particle will be presented here along with possible roles of the core histones in gene regulation, a brief discussion of histone gene organization (particularly H4) and a look at histone H4 protein structure.

The Nucleosome Core Particle

The present understanding of the structure of the nucleosome core particle is the culmination of a wealth of information obtained by the use of various techniques including x-ray and neutron diffraction, electron microscopy, nuclease digestion of chromatin and protein-protein and protein-DNA crosslinking (Bentley et al., 1984; Brandt et al., 1980; Burlingame et al., 1985; Klug et al., 1980; Mirzabekov et al., 1978; Moss et al., 1976; Richmond et al., 1984; and Urban et al., 1979). The nucleosome core particle is made up of an octamer of histones, two each of

histones H2A, H2B, H3, and H4. This core particle has been described by Burlingame and co-workers to a resolution of 3.3A through the use of single crystal x-ray diffraction of chicken erythrocyte histone octamers. The octamer is an ellipsoid 110Å long, 65Å high, and 70Å wide and takes the general shape of a rugby ball. The octamer seems to be divided into three parts, a central tetramer of ($H3_2$-$H4_2$) bounded at each end by an H2A-H2B dimer. This octamer is wrapped with two full superhelical turns of DNA enlarging the diameter to between 100Å and 110Å. Within the two superhelical turns there are approximately 168 base pairs of DNA, 16 helical turns with 10.4 base pairs per turn (Burlingame et al., 1985).

Gene Organization

In the haploid wild type yeast Saccharomyces cerevisiae there are two copies of each of the four core histone genes (figure 1). These genes are located as pairs consisting of one H2A and one H2B gene or one H3 and one H4 gene. The four pairs of genes are unlinked and located at four separate loci. Sequences which code for each of the histone proteins in Saccharomyces cerevisiae have been determined and these DNA sequences have been used to predict the amino acid sequence for each protein. The alleles for H2A and H2B code for two slightly different subtypes of the proteins while both alleles for H3 code for

identical proteins and both alleles for H4 code for identical proteins (Stephenson,1984).

The yeast Saccharomyces cerevisiae is an excellent organism for studying histones because: (1) It contains only 2 gene copies of each of the 4 core histones (most organisms contain multiple copies of the genes making a genetic analysis too complex); (2) The genetics of the species is well documented; (3) It allows the use of the powerful new methods of molecular biology which can be combined with those of genetics; and (4) It is easily grown and maintained in large numbers.

H4 Protein

The present study is concerned with the histone H4 protein in the yeast Saccharomyces cerevisiae. It is a basic protein, 102 amino acids in length with a molecular weight of approximately 11,300 daltons. A large percentage of the protein consists of arginine and lysine residues (14% and 11%, respectively) which result in its net positive charge (Lehninger, 1982).

Five genetically engineered histone H4 mutations have been analyzed to date (Mittman, 1986). These defined mutations are designated hhf1-1, hhf1-d1, hhf1-2, hhf1-3, and hhf1-4 (the abbreviation hhf1 represents: histone H4 copy I, and the number or letter after the dash represents the defined mutation) (figure 2). Three of these mutations

encode nonfunctional histone H4: hhf1-2, hhf1-3, and hhf1-4 (nonfunctional is defined here as the inability to rescue cells which have been deleted for both wild type chromosomal copies of their histone H4 gene). The gene hhf1-2 codes for two amino acid substitutions which replaces glycine with aspartic acid residues at position 13 and 42 thus adding a net overall charge of -2 to the protein. This indicates that one or both of these residues is critical to the functionality of this protein (investigation in process). The hhf1-3 allele encodes 10 amino acid substitutions: glycine is replaced by aspartic acid at positions 10, 14, 48 and 94; arginine by lysine at positions 36, 78 and 92; glutamic acid by lysine at position 52, alanine by threonine at position 76; and glycine by asparagine at position 101. This results in a net overall charge change of only -1, however most of these specific substitutions (positions 10, 14, 48, and 94 glycine by aspartic acid; position 52 glutamic acid by lysine; position 76 alanine by threonine and position 101 glycine by asparagine) involve large changes at their specific position. The third mutation (hhf1-4) found to encode nonfunctional H4 protein, substitutes arginine residues for lysine residues at positions 5, 8, 12, and 16; and a serine for glycine at position 11. This results in a change in structure but the charge at each position remains the same.

The two mutations which provide functional H4 protein are hhf1-d1 encoding an inframe deletion of residues 2-26 and hhf1-1 consisting of the substitution of glutamine for glutamic acid at position 27. The survival of yeast carrying the hhf1-d1 encoded protein is surprising considering its loss of such a large percentage of such highly conserved amino acid residues. Even though the cell is able to survive its growth rate is decreased considerably with this deletion. The single substitution in the hhf1-1 protein adds a +1 charge at position 27 and is evidently not drastic enough to render nonfunctional. Analysis of these mutations has helped accumulate information toward which residues are critical for structure and function, and the effect that certain H4 mutations have on cellular phenotype.

The method used for analyzing these mutations has been tetrad analysis (Mittman, 1986). Yeast can exist in several physiological states (haploid, diploid or spore) and can be transformed with exogenous plasmid DNA. Haploid yeast, deleted for one of the two chromosomal copies of their histone H3-H4 gene set, are transformed with a plasmid carrying a copy of the desired mutant H4 gene. These cells are mated with haploid cells of the opposite mating type which have been deleted for copy II of the H3-H4 gene set and that have also been transformed with a plasmid carrying a copy of the desired mutant H4 gene

(figure 3). The mating results in diploid cells which contain both transformed plasmids as well as a deletion of two of the four allelic copies of H3 and H4 genes.

These diploid cells are induced to sporulate and tetrad spores are teased apart and placed on non selective growth media (YPD). The process is carried out in order to obtain a spore which grows and is deleted for both of its chromosomal copies of the HHT-HHF gene set and thus survives on the plasmid encoded H4 protein mutation. At this point it is impossible to determine what the genotype of each tetrad spore colony is, so a large number of tetrads (approximately 50) must be dissected and placed on YPD agar to grow. Colonies of these cells are used to innoculate nutrient broth in order to grow up large numbers of each type of spore cell so that their DNA can then be retrieved. Through laborious procedures a Southern blot analysis is performed. The DNA is digested with restriction enzymes and electrophoresed on an agarose gel to separate DNA fragments. The DNA is transferred to nitrocellulose and probed with an ^{32}P-labelled H4 gene in order to observe complementary DNA sequences on the nitrocellulose filter. By visualizing the radioactive bands the genotype of each spore cell can be determined and those surviving on mutant H4 gene only, can be isolated.

This series of procedures, used to isolate a cell dependent on the H4 gene encoding mutant histone protein,

can be even more tedious if the mutant H4 protein is nonfunctional. If the mutant is nonfunctional, spores with copy I and copy II deletions will not grow. Therefore large numbers of tetrad spores will have to be analyzed in order to prove statistically that the mutation is nonfunctional. This method, of isolating cells dependent on mutant H4, has hindered the quick analysis of large numbers of these mutations. The purpose of the present work has been to develop a new system ("plasmid shuttle system") for the analysis of histone H4 mutations which will reduce the number of manipulations required therefore making the analysis of large numbers of defined mutations more efficient. The present work has also had the purpose of analyzing a specific histone H4 mutation (hhf1-5) using the newly developed "plasmid shuttle system."

Materials and Methods

Bacteria and yeast strains

The bacteria strain JM83 (Messing and Viera, 1982) used for cloning pBAM11/*hhf1*-5 was obtained from Barbara Mittman's laboratory as was the yeast strain 200*a* used in the plasmid shuttle system. The genotypes for these strains are seen in Table I.

Bacteria JM83 (Messing and Viera, 1982) were grown in L-Broth (1.0% bacto tryptone [Difco], 0.5% bacto yeast extract [Difco] and 0.5% NaCl). Following transformation with pMS301/BAM_o which carries a gene encoding ampicillin resistance, the strain was grown in L-Broth containing 100 ug/ml ampicillin (L-amp). The bacterial cells were plated on, L-Agar (1.0% Bacto tryptone [Difco], 0.5% yeast extract [Difco], 1.0% NaCl and 1.5% agar [Difco] supplemented with 100 ug/ml ampicillin). The bacteria were grown with shaking at 37^{o}C.

Yeast were grown in YPD Agar (1.0% Bacto yeast extract [Difco], 2.0% Bacto peptone [Difco], 2.0% dextrose, and 2.0% Bacto Agar [Difco], and SD Agar (6.7% Bacto yeast Nitrogen Base without amino acids [Difco], 2.0% dextrose, 2.0% Bacto Agar [Difco], with the addition of required amino acids (Sherman et al, 1982). Liquid forms of these media were also used. The yeast were grown at 30^{o}C with shaking for approximately 24 hours.

Restriction enzymes EcoRI, Hind III and T4 ligase were obtained from International Biotechnologies, Inc. (IBI) and used as directed by the manufacturer. Calf intestinal alkaline phosphatase was purchased from Boerhinger Mannheim and used as directed by the manufacturer.

Plasmids pBAM7/BMS001, pMS301/BAM_o , pMS302/476, pBAM10/HHF1, pBAM10/hhf1-2 and pBAM10/hhf1-3 were provided by Dr. Barbara Mittman.

Methods

Plasmid construction

The plasmid pBAM11/hhf1-5 was constructed using the Hind III -EcoRI fragment from pBAM7/BSM001 which was inserted into the Hind III - EcoRI restriction site of pMS301/BAM_o (Figure 4). The insert from pBAM7/BSM001 contains genes for wild type histone H3 and the H4 mutation hhf1-5. Approximately 3 ug of each plasmid (pBAM7/BSM001 and pMS301/BAM_o) were mixed with restriction enzymes EcoRI and Hind III using conditions suggested by the manufacturer. The mixtures were incubated at 37°C for approximately 2 1/2 hours and then heat shocked at 60°C for 10 minutes. Approximately 1/7 of the volume of enzyme cut plasmid pMS301/BAM^o, the total volume of cut pBAM7/BSM001 and a lambda Hind III marker were run on a 1% agarose gel. When the EcoRI and Hind III fragments had separated from the parent plasmid, the electrical current was stopped.

The pBAM7/BSM001 insert band was extracted from the agarose gel using a DEAE cellulose membrane (Schleicher and Schuell). A small piece of DEAE paper was hydrated with deionized water and placed in electrophoresis buffer (40mM Tris base, 20mM sodium acetate, 0.9mM EDTA and 0.6uM ethidium bromide adjusted to pH 8.2). A slit was cut in front of the pBAM7/BSM001 insert band and the piece of DEAE membrane was placed in the slit. The electrical current was reapplied until the band ran onto the membrane paper. The piece of membrane paper containing the DNA band was placed in an Eppendorf tube with 0.5 ml high salt buffer (10mM Tris pH 7.4, 1mM EDTA, 1.5M NaCl) and incubated at 65°c for 15 minutes with intermittent vortexing. One microliter of carrier E. coli tRNA (Sigma, St Louis, Mo) and approximately 1.5 ml of 95% EtOH were added to the mixture and placed at - 70°C for approximately 15 minutes. Following a 15 minute centrifugation in a microcentrifuge, the pellet was washed with 75% EtOH, repelleted, vacuum dried, and redissolved in 10mM Tris EDTA pH 7.0 (TE).

The pMS301/BAM_o plasmid vector previously cut with Hind III was digested with calf intestinal alkaline phosphatase (CIAP). The phosphatase removes 5'phosphate groups from the cut ends of the vector, which are required for the formation of phosphodiester bonds and thus hinders recircularization of the plasmid (Maniatis et al., 1982). One microliter of CIAP was added to approximately 3 ug of

cut pMS301/BAM_o and incubated at 37°C for 30 minutes. Next 180 ul of TE was added along with 200 ul of neutralized phenol. The mixture was vortexed and quickly centrifuged in a microcentrifuge. after centrifugation the top aqueous layer was removed. A 1/10 volume of 3M sodium acetate (pH 5.5) was added and the tube was filled with 95% ethanol and frozen at -70°C for 10 minutes. The mixture was centrifuged for 10 minutes in a microcentrifuge, washed with 75% ETOH and dried.

The phosphatased vector (pMS301/BAM_o) was then cut with EcoRI and added to the BSM001 insert along with ligase. Approximately 400 ng of vector was ligated with approximately 230 ng of insert (giving a molar ratio of approximately 3:1 insert to vector). A positive control containing vector religated in sterile H_2O was also performed.

Bacterial transformation

Bacterial cells JM83 (Messing and Viera, 1982) were made competent for transformation by first growing in L-broth overnight. Next 25ml of L-Broth was inoculated with approximately 0.25 ml of the overnight culture and allowed to grow for 2 1/2 hours at 37°C. The cells were centrifuged at approximately 3,300xg for 10 minutes in a SS34 rotor using a Servall centrifuge. The pellet was resuspended in 12.5 ml of 0.1M $MgCl_2$. The cells were

recentrifuged, the supernatant was then poured off, and the pellet was resuspended in 1.2 ml of 0.1 M $CaCl_2$. The cells were placed on ice for 30 minutes in order to allow them to become competent.

Next 0.2 ml of the competent cells were added to five different test tubes for transformation. Two of the test tubes of competent cells received 17 ul of the ligase, vector insert solution (pBAM7/BMS001 insert and $pMS301/BAM_o$ cut with EcoRI and Hind III). Positive controls were one tube which received undigested $pMS301/BAM^o$ in sterile H_2O and one tube containing $pMS301/BAM_o$ cut with enzymes EcoR1 and HindIII but without the pBAM7/BSMOO1 insert. Linear DNA does not efficiently transform competent cells. The last tube, the negative control had only competent cells. Each of the five tubes were placed on ice for 30 minutes and then heat shocked at 37^oC for 5 minutes. Following the addition of 0.8 ml L-broth and a 20-30 minute incubation at 37^oC, the cells were plated on L-amp plates.

Colonies were selected from the experimental plates and each was used to inoculate 2.0 ml of L-amp Broth. The cells were grown in L-amp broth for 5 - 6 hours at 37^oc. These cultures were used to make plasmid mini-DNA preparations.

For mini DNA preparations, 1.5 ml of each cell culture were poured into an Eppendorf tube and centrifuged for one minute in a microcentrifuge. The supernatant was poured

off and the pellet was resuspended in 100 ul of a 50 mM glucose, 10 mM EDTA, and 25 mM Tris-Cl solution (pH 8.0.) After the solution was stored at room temperature for 5 minutes 200 ul of a 0.2 N NaOH 1% SDS solution was added. The tubes were mixed by inversion and placed on ice for 5 minutes. A 150 ul volume of 3 M sodium acetate (pH 5.5) was added. The solution was vortexed and again placed on ice for 5 minutes. The solution was centrifuged in a microcentrifuge and the supernatant was transferred to a new tube. The DNA was phenol extracted by adding an equal volume of phenol/chloroform, vortexing, centrifuging for 2 minutes and transferring the supernatant to a new tube. The DNA was washed with 2 volumes of 95% ethanol, centrifuged for 5 minutes in a microcentrifuge, and the supernatant was poured off. One ml of 75% ethanol was added to the pellet which was vortexed and recentrifuged. The supernatant was removed and the pellet was allowed to air dry.

The dried pellets were resuspended in 30 ul of TE and then divided into 2 tubes (one experimental and one control). The experimental tube was digested with EcoRI and Hind III. One tube was left undigested as a control. Samples of each tube were run on an electrophoresis gel along with a ladder of known band sizes obtained from bacteriophage lambda DNA digested with Hind III.

Large Scale Plasmid Preparation

A bacterial colony containing the desired pBAM11/hhf1-5 plasmid was used to start an overnight culture in 20 ml L-amp broth. Four ml of this overnight culture was used to inoculate 400 ml of fresh L-amp broth. Two ml of a 34 mg/ml solution of chloramphenicol in 95% ethanol was added to the culture after growing for 3 - 4 hours. The culture was grown for another 16 hours and the cells were pelleted at 6000 RPM or at a RCF of approximately 4000xg in a GSA rotor wwith a Servall centrifuge. The supernatant was poured off and the pellets were resuspended in 4 ml of 25% sucrose in 50 mM Tris (pH 7.5).

All cell lysis procedures were performed on ice in order to decrease DNA degradation by nucleases. The bacterial cell walls were weakened with a 5 minute incubation with 2 ml of a 10 mg/ml lysozyme solution followed by a 5 minute incubation with 2 ml of 0.5M EDTA pH 8.0. Cells were lysed during a 20 minute incubation with 5.0 ml of lysis mix (5 ml of 0.1% Triton x-100, 62 mM EDTA, 50 mM Tris pH 8.0). The solutions were centrifuged in a Servall centrifuge and a SS34 rotor at approximately 13,000xg for 40 minutes and then the supernatant was mixed with an equal number of grams of cesium chloride. A 1/10 volume of 5mg/ml ethidium bromide stock solution was added and the solutions were placed in ultracentrifuge tubes, capped and centrifuged for 40 hours in a Beckman model L3-

50 ultracentrifuge and a Ti50 rotor at 38,000 RPM or a RCF of approximately 109,000xg. DNA bands were visible with short wavelength ultra violet light and were collected by puncturing the ultracentrifuge tube using a needle and extracting the DNA with a syringe. The DNA was dialyzed for one hour in TE and then phenol extracted and dialyzed for 16 hours with one change of the TE buffer after 8 hours.

Yeast Transformations

A single colony of the desired yeast strain was used to inoculate YPD for an overnight saturated culture. The overnight culture was then used to inoculate 200 ml of YPD, which was allowed to grow to approximately 1.5 x 10^7 cells/ml. Cells were centrifuged for 5 minutes in a GSA rotor, at 6000 RPM or at a RCF of approximately 4000xg, in a Servall centrifuge. The cell pellet was washed twice with TE and then repelleted. The pellet was resuspended in 20 ml of 0.1 M lithium acetate in TE, incubated for one hour at 30^{o}C, and repelleted. Finally, the supernatant was removed and 2.5 ml of a 0.1 M lithium acetate, 15% glycerol in TE was added to the pellet.

To transform the cells, 1-5 ug of the desired plasmid vector and 0.7ml of 50% PEG 4000 was added to 0.3 ml of competent cells and incubated at 30^{o}C for one hour. One set of competent cells did not receive any plasmid DNA and served as a negative control. The cells were heat shocked

at 42°C for 5 minutes and centrifuged for approximately 2 seconds. The pellet was washed with minimal media (SD) resuspended in SD and plated on a SD plus adenine, lysine, and leucine agar plate.

Statistical test of data

A statistical test was performed (hypothesis test for two population proportions (Weiss and Hasset, 1987) to demonstrate that the difference between two population proportions (given below) was not just due to sampling error.

The first population proportion consisted of the proportion of cells obtained which possessed two plasmids (pBAM10/HHF1 and pMS302/476) each encoding wild type H4 and which lost the plasmid selected against (pBAM10/HHF1). The second population proportion was the proportion of cells obtained which passed two plasmids, pBAM10/HHF1 encoding wild type H4 and pBAM11/hhf1-5 encoding mutant H4, and which lost the plasmid selected against (pBAM10/HHF1) (table II).

Results

Development of the plasmid shuttle system

A new plasmid shuttle system was developed for testing histone H4 mutations in yeast. This system will make the analysis of large numbers of defined mutations more efficient than the previous method of tetrad analysis. The plasmid shuttle system relied on the availability of plasmid dependent haploid yeast cells (strain 200a). A plasmid dependent cell is defined as a cell deleted for both chromosomal copies of their essential histone H4 gene (copy I and II) and surviving on wild type histone H4 protein encoded on a plasmid. The yeast cells used in the initial development of the system, were dependent on the plasmid pMS302/476 (figure 5.0). Plasmid pMS302/476 contains wild type histone H3 and H4 genes, a gene (ura-3) and several other DNA fragments including a cloned centromere and an autonomously replicating sequence (ARS) (figure 6). The cloned centromere and ARS allow this plasmid to function as a minichromosome during meiosis and mitosis. The ura-3 gene allows the correction of the block in the biosynthetic pathway for uracil. The yeast strain without plasmid genes contains a defect in its ability to produce the pyrimidine base uracil and the amino acid tryptophan. In order for the cells to survive they must

have the amino acid tryptophan and the pyrimidine base uracil available, either in the growth medium or have the biosynthetic block corrected by a plasmid born wild type copy of the gene correcting the chromosomal nutritional defect. These yeast cells are able to synthesize their own uracil because of the presence of the uracil gene on the pMS302/476 plasmid. These cells, which are capable of growing without uracil provided in the growth media, were transformed with another plasmid, pBAM10/HHF1 (figure 5.1). This plasmid contains a gene which allows tryptophan production (allows the correction of the block in the biosynthetic pathway to tryptophan), genes for wild type H3 and H4 as well as the centromere and ARS sequences (figure 6).

The transformed yeast cells were then grown on media lacking both uracil and tryptophan (selective media). Although both plasmids contain a cloned centromere these minichromosomes are still orders of magnitude less stable than normal yeast chromosomes during mitosis. If cells are not dependent on one of these plasmids it can be lost from the cell at a frequency of approximately 1 out of every 100 cells (Fitzgerald-Hayes et al., 1982; Stinchomb et al., 1982; Murray and Szostak, 1983). The cell must retain one of the plasmids to code for histone H4 protein since the chromosomal copies of the gene are deleted.

In an attempt to lose plasmid pMS302/476 the cells

(grown overnight in defined liquid media lacking tryptophan) were plated on solid media containing uracil but lacking tryptophan (uracil (+) tryptophan (-)). Therefore the plasmid correcting the uracil deficiency (pMS302/476) could be lost from a cell while maintaining selection for pBAM10/HHF1 (figure 5.2). In order to determine those cells which had lost pMS302/476, the petri dish containing the cell colonies growing on tryptophan deficient media, was replica plated onto solid media lacking both uracil and tryptophan (uracil (-) tryptophan (-)) (figure 5.3). Those colonies which grew on the initial uracil (+) tryptophan (-) dish but not on the uracil (-) tryptophan (-) dish must contain only pBAM10/HHF1 having lost pMS302/476. These cells were unable to grow on uracil (-) tryptophan (-) media because they could no longer synthesize their own uracil. The frequency of obtaining cells which lost the pMS302/476 plasmid is shown in table II.

The sequence of events described was a test of the plasmid shuttle system. The system made it possible to shuttle an *in vitro* constructed plasmid, containing a mutant H4 gene instead of the wild type gene , into cells dependent on plasmid wild type H4. The next step involved promoting the loss of the plasmid encoding wild type H4 thus making it possible to isolate cells which are surviving only on mutant H4 (that is if the mutation is not

lethal). If a mutation was analyzed that resulted in the synthesis of nonfunctional H4 protein the same growth would have occurred on uracil (-) tryptophan (-) media as on uracil (-) tryptophan (+). The cells would be dependent on the plasmid containing the wild type H4 gene and thus it could not be lost from the cell and allow cell survival.

In all experiments using cells initially dependent on pBAM10/HHF1 (as opposed to pMS302/476) cells were grown for 5 nights as opposed to one when attempting to lose pBAM10/HHF1. This was accomplished by transfering 40ul from each previous overnight culture into 10ml of fresh liquid media (uracil (-) tryptophan (+)). The increased growth period was required because of the lower frequency of plasmid loss under these conditions.

Several control experiments were run using plasmids containing mutations previously examined and yeast cells dependent on pBAM10/HHF1 (pBAM10/HHF1 contains a gene coding for wild type H4). The result of each experiment with the plasmid shuttle system is seen in table II.

Five different trials were run, using five different plasmid combinations in order to test and demonstrate the usefulness of this system. Column one (table II) gives the results of the initial test of the system, as described above, using cells dependent on pMS302/476 and then transforming in pBAM10/HHF1. Both of these plasmids contain a gene encoding wild type H4 protein. They were

used in the initial test of the system because both plasmids are capable of supporting a cell without the presence of the other. In the initial test of the system this was required so that the dependency of the cell for each plasmid could be controlled by the addition or subtraction of the vital nutrients encoded by the plasmids rather than the plasmids ability to provide functional H4 protein. Column 2 gives the results of the reverse shuttling of the same plasmids seen in column 1. In this situation cells dependent on pBAM10/HHF1 were transformed with pMS302/476 in an attempt to lose pBAM10/HHF1. The final three columns give the results of using cells dependent on pBAM10/HHF1 and which have been transformed with plasmids carrying a mutated histone H4 gene. Column 3 gives the results of the trial using a newly constructed plasmid carrying the H4 gene mutation (hhf1-5) never before examined in the cell. Column 4 and 5 give the results of trials using plasmids containing H4 gene mutations previously examined using the old system described in materials and methods (tetrad analysis). The mutations are hhf1-2 and hhf1-1 and encode nonfunctional and functional H4 protein respectively.

A plasmid was constructed here (pBAM/hhf1-5) to analyze a laboratory constructed H4 gene mutation never before examined (figure 4). The plasmid contains the gene coding for the mutation, a gene coding for uracil

production and as well the ARS, and centromere sequences. Yeast cells dependent on pBAM10/HHF1 (the isolated cells from the system initial test) were transformed with this plasmid and the remaining steps of the plasmid shuttle system were carried out as described for the initial test of the system (figure 7).

The hypothesis test for two population proportions at a 5% significance level provided sufficient evidence to conclude that the proportion of cells which carried wild type H4 on both plasmids and lost pBAM10/HHF1 (10.68%) was different and greater than the proportion of cells losing pBAM10/HHF1 having a wild type H4 gene on one plasmid and the mutant H4 on the other (0%)(table II).

Discussion

Each combination of plasmids tested in the plasmid shuttle system had a specific purpose in demonstrating the usefulness of the new system. Experiment 1 (pMS302/476 and pBAM10/HHF1) demonstrated that the system makes it possible to isolate cells dependent on a plasmid newly transformed into the cell (pBAM10/HHF1) and to lose the plasmid on which the cell was initially dependent pMS 302/476. The results indicate that 92.68% of the cells initially containing both plasmids can lose the unneeded plasmid when grown under nonselective conditions. Thus the cells will be surviving on the histone H4 protein encoded by the new plasmid (pBAM10/HHF1).

Experiment 2 demonstrates the reverse of the shuttling in trial 1. In this trial cells dependent on pBAM10/HHF1 are transformed with pMS302/476. Plasmid pBAM10/HHF1 is lost from 10.68% of the cells after growing on selective nutrient media. These results showed that the system could work in either direction. However plasmid loss in the reverse direction occurred at a much lower frequency. This may be due to several factors including differences in overall DNA sequences between the 2 plasmids or use of different cloned centromeres (ie. centromere III verses centromere IV). The two plasmids can be shuttled in and out of the chromosomal H4 gene deleted cells in any order as long as one of them remains to encode essential histone H4 protein. Experiment 3 was performed in order to examine a histone H4 gene mutation never before examined in the cell (hhf1-5). The results strongly indicate that the mutation does not encode functional H4 protein (table II). Plasmid pBAM10/HHF1 dependent cells transformed with pMS302/hhf1-5 when grown on selective media (uracil (-) tryptophan (+)) were unable to lose pBAM10/HHF1. The hypothesis test for two population proportions (10.68% from trial 2 and 0% from column 3) gave sufficient evidence to conclude that the frequency of plasmid pBAM10/HHF1 loss was greater in trial 2 than trial 3 (table II). Thus the at least 10-fold difference in the frequency of plasmid loss is not due to sampling error and can be expected to occur

again under the same conditions.

Experiment 4 and 5 were used to test the system using histone H4 gene mutations which have a known effect on the cell (hhf1-1 and hhf1-2 provide functional or nonfunctional H4 protein respectively). Experiment 4, shuttling pMS302/hhf1-2 into cells dependent on pBAM10/HHF1, showed that none of the cells were able to lose pBAM10/HHF1. This was expected since the hhf1-2 allele does not encode functional H4 protein therefore the cells are still dependent on pBAM10/HHF1 for functional histone H4 protein.

Experiment 5 also used cells dependent on pBAM10/HHF1 and transformed them with pMS302/hhf1-1. The results show that 30.02% of the cells grown on selective media were able to lose pBAM10/HHF1. The ability of these cells to lose the original plasmid coding for wild type H4 protein was expected since the hhf1-1 allele transformed into the cell is capable of providing functional H4 protein. The large percentage of cells losing pBAM10/HHF1 was somewhat unexpected when compared to the 10.68% seen in trial 2. The cause of this difference is unknown however the difference may reflect a mild selection for cells not carrying any histone H4 mutations.

These trials have demonstrated that the new system is successful in isolating cells which code for mutant H4 protein only and determining whether a specific mutation provides for functional H4 protein. If a mutation does not

encode functional histone H4 protein then no cells, deleted for both of their chromosomal H4 gene copies, will be able to survive without a plasmid coding for functional H4 protein. Thus, in the newly developed system, no cells will lose the original plasmid containing the gene encoding wild type H4 protein (pBAM10/HHF1).

The newly examined (hhf1-5) H4 protein mutation consists of six amino acid substitutions: arginine is replaced by lysine at position 3, glycine by aspartic acid at position 6 and 42, glycine by serine at position 7 and 41, and alanine by threonine at position 38. This results in a net overall charge of -2 (as well as the polar-nonpolar alteration at position 38). In the previously tested hhf1-2 mutation, the substitutions of glycine with aspartic acid at position 13 and 42 made the protein nonfunctional. The mutation hhf1-5, analyzed here, also has a substitution of glycine by aspartic acid at these positions. The change at position 42 is currently being tested to determine whether this change alone is responsible for the nonfunctionality of the H4. Since numerous changes occur throughout the hhf1-5 allele it was not expected to encode functional protein.

The development of this new system will make it possible to screen large numbers of histone H4 gene mutations much more easily and quickly than the old tetrad analysis method. By screening large numbers of specific H4

mutations it will be possible to determine specific residues which are critical to the function of the protein. This information will lead to a better understanding of the three dimensional structure of histone H4, its interactions with other histones and related functions in the cell.

The present research has been to develop a rapid new system for analyzing histone H4 mutations in yeast and to use the system to analyze a specific *in vitro* constructed H4 gene mutation. The system development has been successful and the system has been used to determine that the *hhf1-5* allele does not encode functional histone H4 protein.

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Table I

Strain	Phenotype	Reference
Bacteria		
JM83	ara △(lac pro)thi lac Z △M15	Messing and Vieira, 1982
Yeast		
200	a △(HHT1-HHF1) △(HHT2-HHF2) ura3-52 ade2-101 lys2-801 trp1-289 and dependent on pMS302/476	B. A. Mittman, 1986

Table II

Experiment #	1	2	3	4	5
Plasmids involved	pMS302/476 pBAM10/*HHF1*	pBAM10/*HHF1* pMS302/476	pBAM10/*HHF1* pBAM11/ *hhf1*-5	pBAM10/*HHF1* pMS302/ *hhf1*-2	pBAM10/*HHF1* pMS302/ *hhf1*-1
Plasmid attempting to lose	pMS302/476	pBAM10/*HHF1*	pBAM10/*HHF1*	pBAM10/*HHF1*	pBAM10/*HHF1*
Frequency of cells losing plasmid	$\frac{38}{41}$	$\frac{25}{234}$	$\frac{0}{198}$	$\frac{0}{161}$	$\frac{127}{423}$
Percent of cells losing plasmid	92.68	10.68	0	0	30.02
Conclusion about H4 gene	wild type	wild type	nonfunctional	nonfunctional	functional
Statistical test conclusion	An hypothesis test for two population proportions gave sufficient evidence to conclude that the frequency of plasmid loss for experiment #2 (25/234) was greater than that in experiment #3 (0/198). At a 5% significance level (alpha = 0.05) the null hypothesis was rejected.				

Figure 1. Organization of the histone genes in the yeast Saccharomyces cerevisiae. There are two copies of each gene H3, H4, H2A, and H2B. They are found as unlinked gene sets (H3 and H4) and (H2A and H2B). Restriction enzyme sites for EcoR1 (E) and HindIII (H) are found at the location of these gene sets.

Histone gene organization in S. cerevisiae

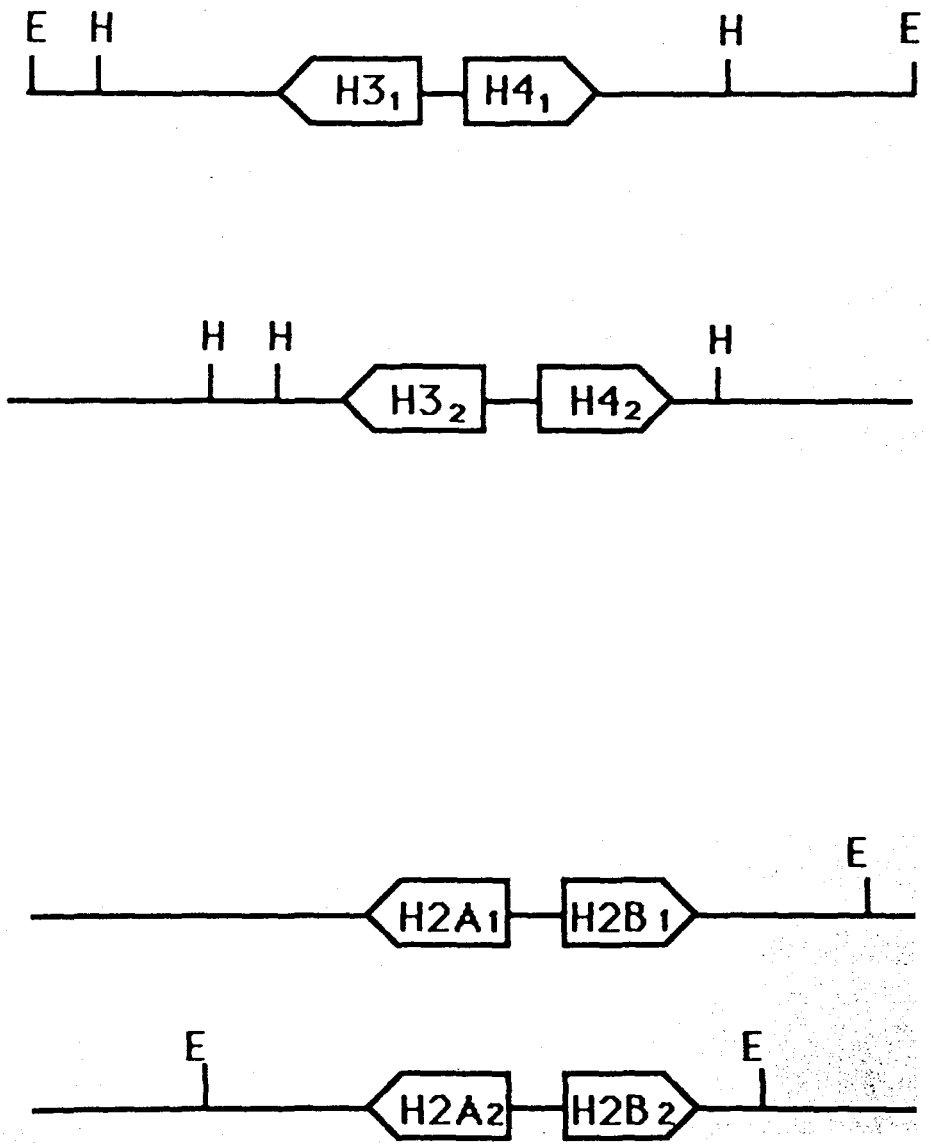


Figure 2. Amino acid sequence of wild type yeast histone H4 (HHF1) and laboratory constructed H4 substitutions. Substitution are represented by an asterisks.

A = Alanine	L = leucine
R = Arginine	K = Lysine
N = Asparagine	M = Methionine
D = Aspartic acid	F = Phenylalanine
C = Cysteine	P = Proline
Q = Glutamine	S = Serine
E = Glutamic acid	T = Threonine
G = Glycine	W = Tryptophan
H = Histidine	Y = Tyrosine
I = Isoleucine	V = Valine

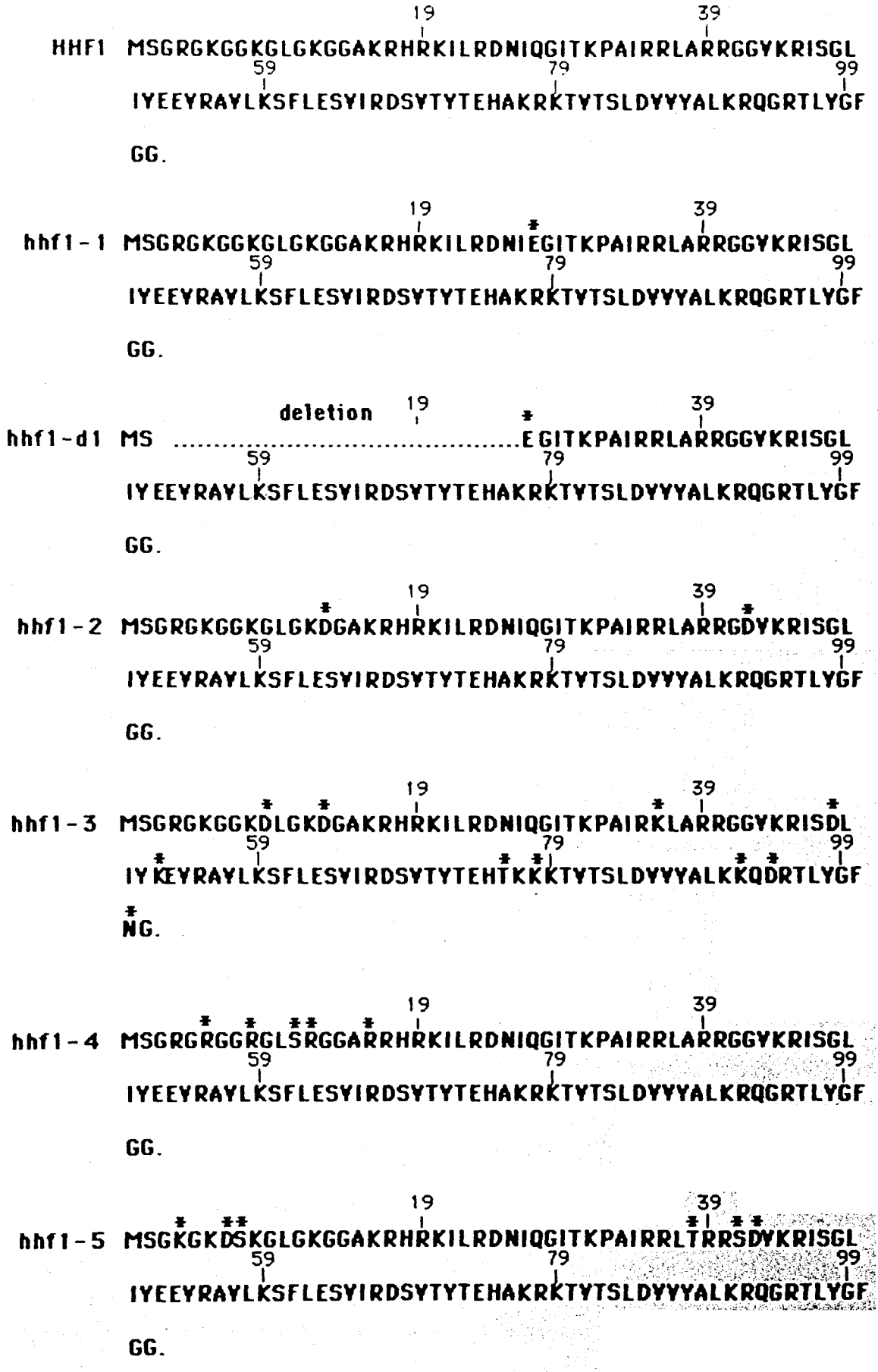

HHF1 MSGRGKGGKGLGKGGAKRHRKILRDNIQGITKPAIRRLARRGGVKRISGL
IYEEYRAVLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGF
GG.
hhf1-1 MSGRGKGGKGLGKGGAKRHRKILRDNIEGITKPAIRRLARRGGVKRISGL
IYEEYRAVLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGF
GG.
deletion
hhf1-d1 MS .....EGITKPAIRRLARRGGVKRISGL
IYEEYRAVLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGF
GG.
hhf1-2 MSGRGKGGKGLGKDGAKRHRKILRDNIQGITKPAIRRLARRGDVKRISGL
IYEEYRAVLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGF
GG.
hhf1-3 MSGRGKGGKDLGKDGAKRHRKILRDNIQGITKPAIRKLARRGGVKRISDL
IYKEYRAVLKSFLESYIRDSYTYTEHTKKKTYTSLDYYYALKKQDRTLYGF
NG.
hhf1-4 MSGRGRGGRGLSRGGARRHRKILRDNIQGITKPAIRRLARRGGVKRISGL
IYEEYRAVLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGF
GG.
hhf1-5 MSGKGKDSKGLGKGGAKRHRKILRDNIQGITKPAIRRLTRRSDVKRISGL
IYEEYRAVLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGF
GG.

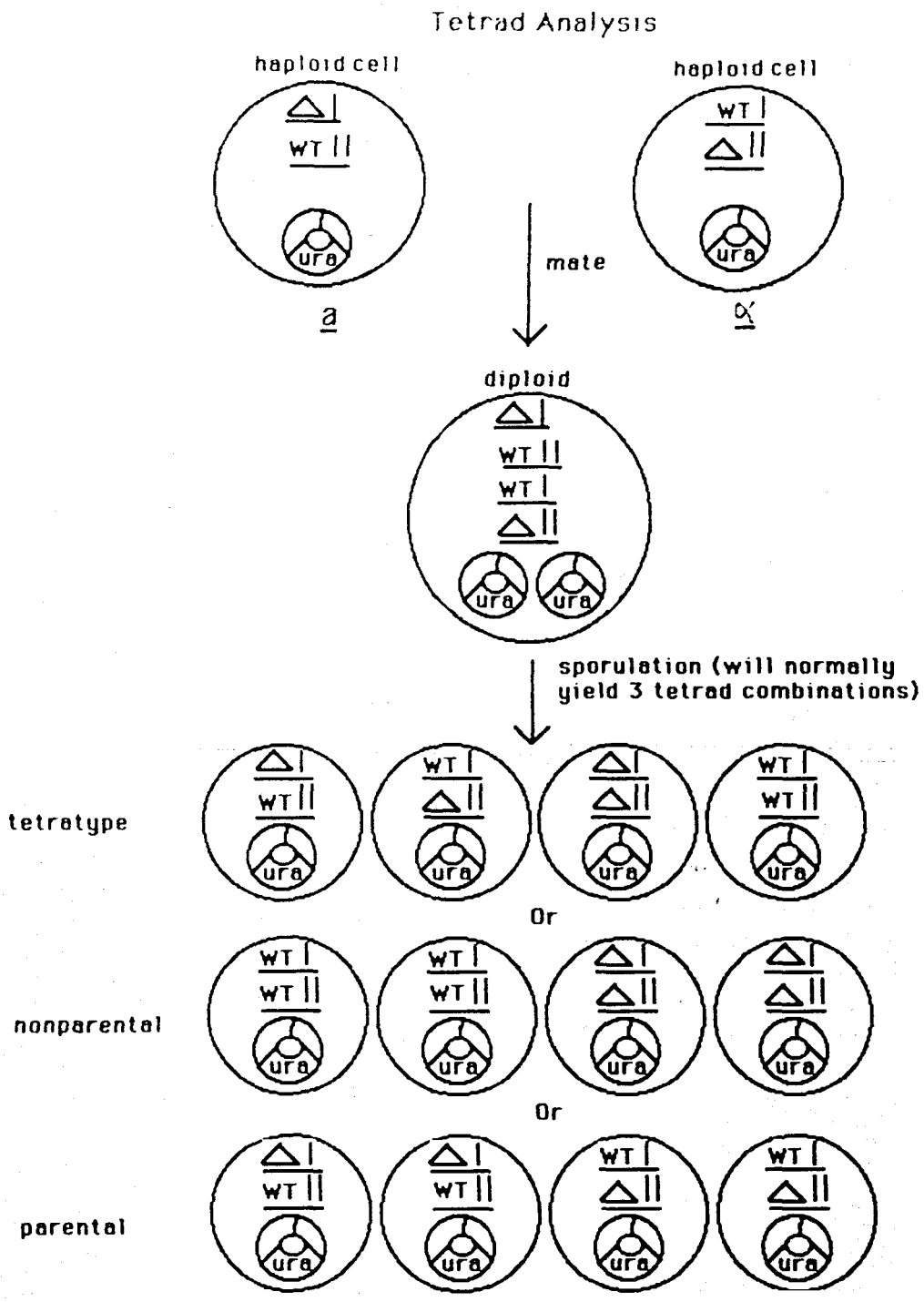
Figure 3. Procedures involved in the isolation of cells surviving on mutant histone H4 by tetrad analysis.


= deletion of gene

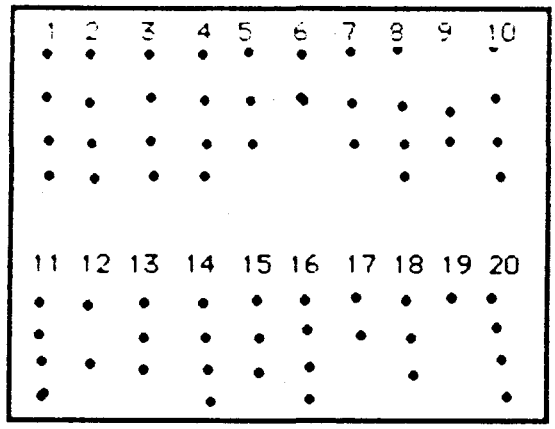
I and II = copy I and copy II of histone H3 and H4 genes

WT = wild type histone H3 and H4 genes

= plasmid carrying a gene which allows for the production of uracil and encodes the desired H4 mutation


Tetrad Analysis
haploid cell
haploid cell
WT I
Δ II
ura
mate
a
α
diploid
WT II
WT I
Δ II
ura
ura
sporulation (will normally yield 3 tetrad combinations)
tetratype
nonparental
parental
Or
Or

tetrad spores are teased apart and grown on YPD.



Agar slab with sets of tetrad spores (note not all spores will grow into colonies).

Each spore colony is grown up in large number in a liquid medium and the DNA is retrieved.

The DNA is cut with restriction enzymes at sites on either side of the H4-H3 gene set, and fragments are separated by gel electrophoresis.

The DNA is transfered to nitrocellulose and probed with a radioactively labeled complementary DNA fragment to the HHF1 gene. By visualizing the radioactive bands the genotype of each spore can be determined.

Figure 4. The construction of pBAM11/*hhf1*-5 from pMS301/BAM° and pBAM7/BSM001 fragments.

Restriction enzyme sites:

E = *EcoR*1

H = *Hind*III

B = *BAM*H1

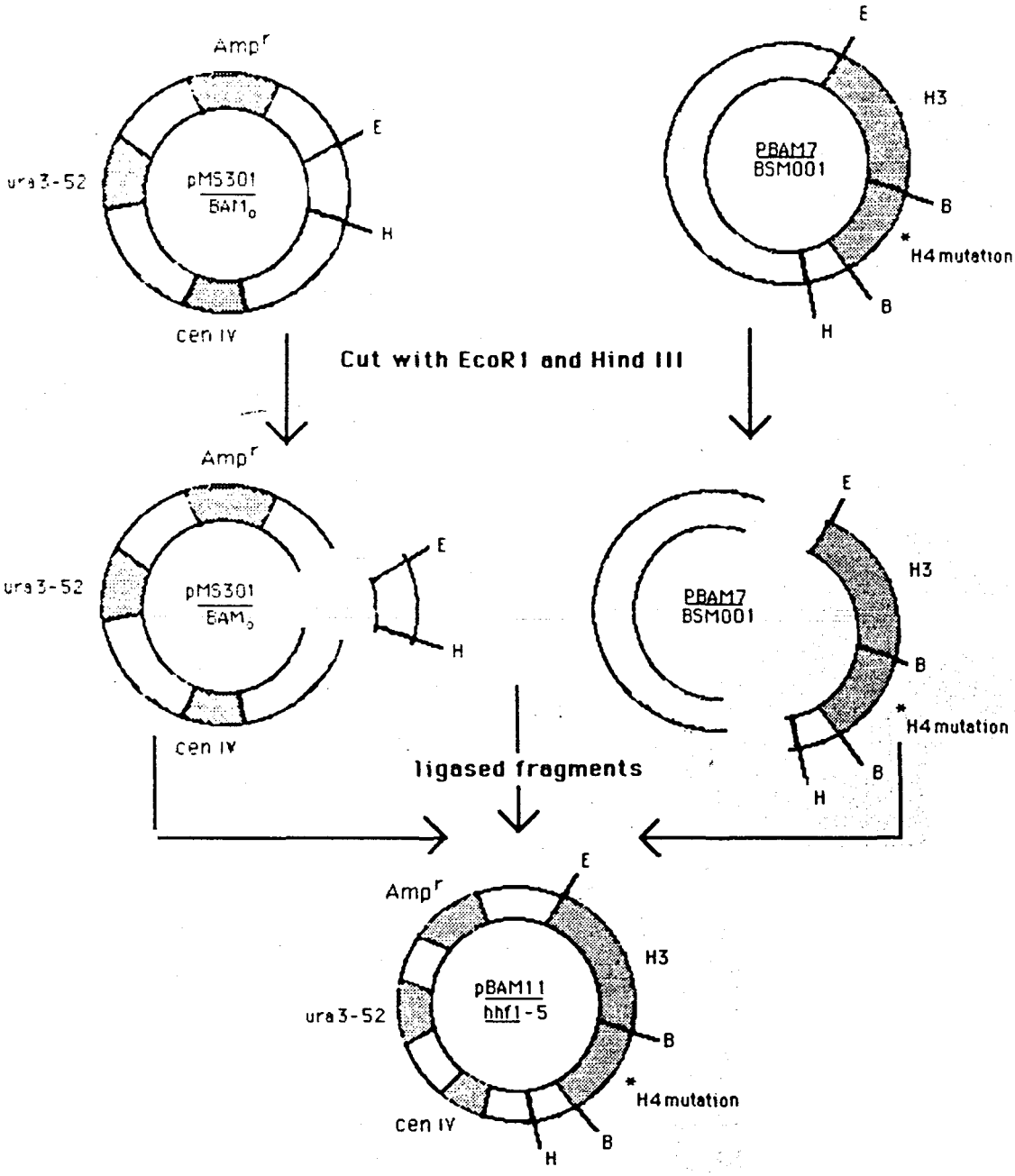

Amp^r
ura3-52
pMS301
BAM_o
E
H
cen IV
E
H3
PBAM7
BSM001
B
*H4 mutation
B
H
Cut with EcoR1 and Hind III
Amp^r
ura3-52
pMS301
BAM_o
E
H
cen IV
E
H3
PBAM7
BSM001
B
*H4 mutation
B
H
ligased fragments
Amp^r
ura3-52
pBAM11
hhf1-5
E
H3
B
*H4 mutation
cen IV
B
H

Figure 5.0-5.3. Representation of the initial test of the plasmid shuttle system.

△	= deletion of gene
I	= copy I of histone H3 and H4
II	= copy II of histone H3 and H4 genes
WT	= Wild Type histone H3 and H4 genes
cen	= cloned centromere

ura and trp	= genes allowing the production of uracil or tryptophan
ura^+ and ura^-	= the ability and the lack of ability respectively of the cell to produce uracil
trp^+ and trp^-	= the ability and the lack of ability respectively for the cell to produce tryptophan

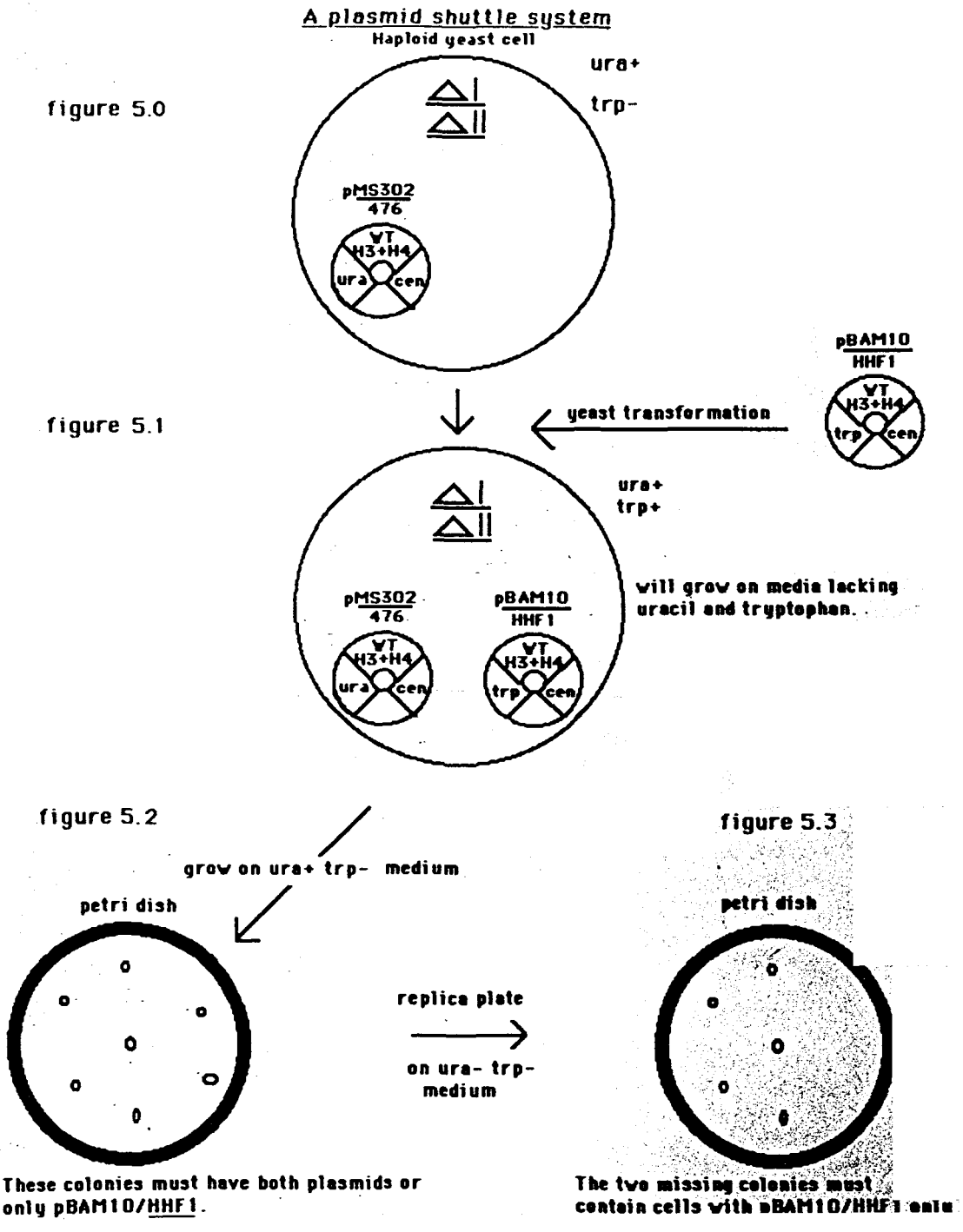

A plasmid shuttle system
Haploid yeast cell
ura+
trp-
pMS302
476
WT
H3+H4
ura
cen
figure 5.0
figure 5.1
pBAM10
HHF1
WT
H3+H4
trp
cen
yeast transformation
ura+
trp+
will grow on media lacking uracil and tryptophan.
pMS302
476
pBAM10
HHF1
figure 5.2
grow on ura+ trp- medium
petri dish
replica plate
on ura- trp- medium
figure 5.3
petri dish
These colonies must have both plasmids or only pBAM10/HHF1.
The two missing colonies must contain cells with pBAM10/HHF1 only

Figure 6. Structure of plasmids pBAM10/HHF1 and pMS302/476 indicating pertinent genes and restriction enzymes sites.

Genes:

ars = autonomously replicating sequence

cen = cloned centromere

Ampr = gene allowing bacterial resistance to ampicillin

ura3-52 = gene allowing uracil production

trp1-289 = gene allowing tryptophan production

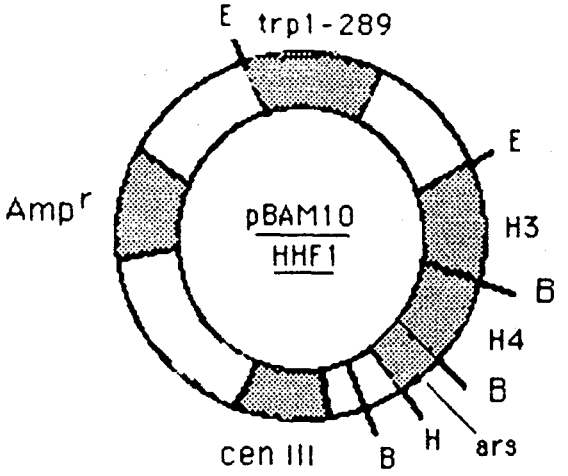
H3 and H4 = genes encoding wild type histone H3 and H4

Restriction enzyme sites:

E = EcoR1

H = HindIII

B = BAMH1


E
trp1-289
E
Amp^r
pBAM10
HHF1
H3
B
H4
B
H
ars
cen III
B

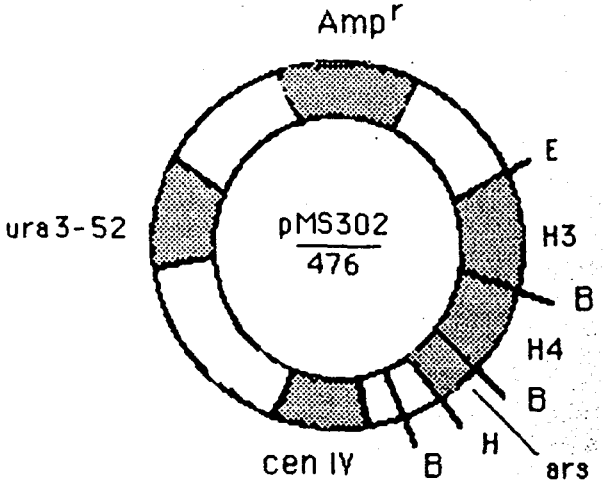
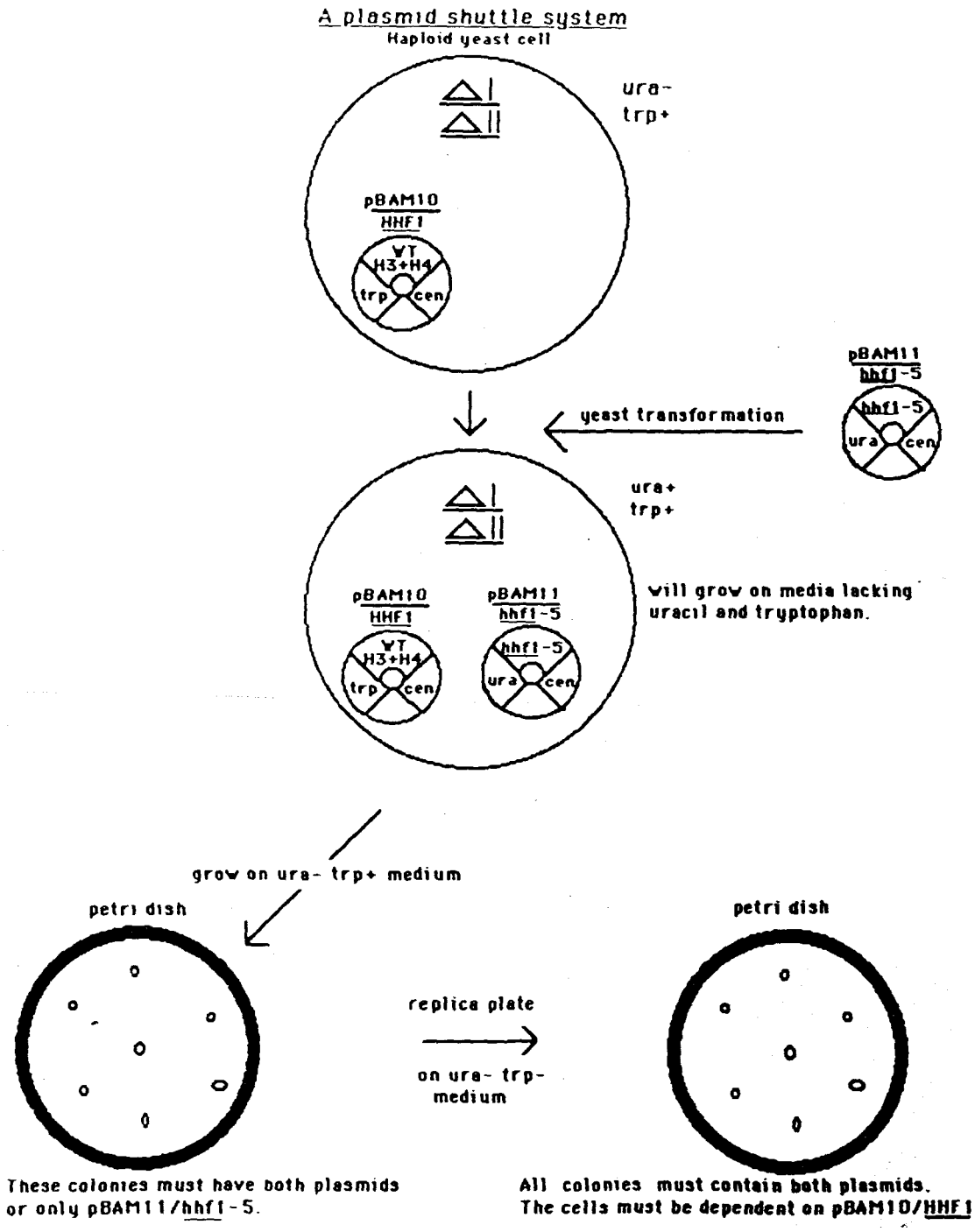

Amp^r
E
ura3-52
pMS302
476
H3
B
H4
B
H
ars
cen IV
B

Figure 7. Use of the plasmid shuttle system in the analysis of the histone H4 mutant <u>hhf1</u>-5.

$\triangle$	= deletion of gene
I	= copy I of histone H3 and H4 genes
II	= copy II of histone H3 and H4 genes
WT	= wild type histone H3 and H4 genes
cen	= cloned centromere

ura and trp	= genes allowing the production of uracil or tryptophan
ura^+ and ura^-	= the ability and lack of ability respectively for the cell to produce uracil
trp^+ and trp^-	= the ability and lack of ability respectively for the cell to produce tryptophan


A plasmid shuttle system
Haploid yeast cell
ura-
trp+
pBAM10
HHF1
WT
H3+H4
trp
cen
pBAM11
hhf1-5
hhf1-5
ura
cen
yeast transformation
ura+
trp+
pBAM10
HHF1
WT
H3+H4
trp
cen
pBAM11
hhf1-5
hhf1-5
ura
cen
Will grow on media lacking uracil and tryptophan.
grow on ura- trp+ medium
petri dish
replica plate
on ura- trp- medium
petri dish
These colonies must have both plasmids or only pBAM11/hhf1-5.
All colonies must contain both plasmids. The cells must be dependent on pBAM10/HHF1

Vita

Edward Rex Lakey Harkrader was born in Richmond, Virginia in 1963. He resided in Louisa county, Virginia where he also attended public schools through high school. He received a Bachelor of Arts degree in biology in May 1986 from the University of Richmond in Richmond, Virginia. In June, 1988 he finished the requirements for his master's degree in biology, at the University of Richmond. In August, 1988 he will begin work on his M.D. at the Medical College of Virginia.