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Investigating the viability of yeast expressing histone from extrachromosomal DNA : progress in construction of a plasmid bearing one copy of each yeast histone gene

Ralph Donaldson Hellams Jr.

ABSTRACT

Much of the current information on histones has been based on *in vitro* studies. The original goal of this research was to construct a mutant strain of *Saccharomyces cerevisiae* containing all chromosomal copies of the yeast histone genes made nonfunctional. This project would demonstrate whether such a strain could be rescued by a plasmid carrying the wild-type copies of the four core histone genes. Furthermore, this yeast strain construct would allow future investigations to take advantage of histone mutant analysis *in vivo*. The critical step in beginning this work was the construction of a plasmid which contained the genes coding for histones H2A, H2B, H3, and H4. Construction of this plasmid proved difficult to complete due to such problems as using a nontransformable bacterial single colony, utilizing a plasmid which was subsequently found to be in fact another plasmid.

This thesis describes specific methods used for transformation, partial digestion and DNA recovery techniques which were attempted and the results of these attempts. The approaches developed should simplify future construction of the plasmid and contribute to further studies investigating the viability of yeast using histone expressed from extrachromosomal DNA.

INVESTIGATING THE VIABILITY OF YEAST EXPRESSING HISTONE FROM EXTRACHROMOSOMAL DNA : PROGRESS IN CONSTRUCTION OF A PLASMID BEARING ONE COPY OF EACH YEAST HISTONE GENE

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Dedication

This work is dedicated to the memory of my sister, my friend, Dr. Susan Elizabeth Hellams, M.D.

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Current Model Systems

Numerous biochemical and physical studies have been performed on the nucleosome and the nucleosome core particle to better understand this chromatin component. To date, no naturally occurring histone mutations have been found in yeast, unlike the case for most eukaryotic proteins. From experiments using specific genetic altering of prescribed regions of individual or paired histones, greater insight should be gained regarding the precise role histones play in the cell.

There are two genetic approaches currently used in studying histones. The reverse genetic approach focuses on engineering a specific genetic mutation within a given histone gene *in vitro* followed by an analysis of any phenotypic alterations that result. The alternative forward genetic approach relies on subjecting whole cells to some mutagenic agent such as ultraviolet light, observing any phenotypic changes that arise from the procedure and then identifying the cause of the new phenotype as being due to a histone mutation. Through such genetic investigations combined with studies from x-ray diffraction, a clearer understanding of histone function should emerge. To date, little molecular research has been performed on histone H3,

although some investigations are beginning to concentrate on this histone (M. M. Smith, personal communication). Conversely, numerous studies have been performed on histones H2A, H2B and H4 (Schuster, et al., 1986; Wu, et al., 1987; McGee, et al., 1990). Frameshift mutations within the two histone H2B genes (H2B1 and H2B2) have been introduced into Saccharomyces cerevisiae by transformation and recombination (Rykowski, et al., 1981). This work showed that mutant strains carrying either the H2B1 or H2B2 frameshift mutation are viable and can undergo all phases of the yeast life cycle. Thus yeast cells lacking one of the H2B variants can survive. However, strains bearing mutations in both H2A and H2B germinate, bud only once and are incapable of further growth. Later studies only utilized a background lacking wild-type H2B proteins (Wallis, et al., 1983). These experiments revealed that although the amino termini of the two H2B histones are highly conserved through evolution, deleting large segments of the N-terminus of H2B does not affect the viability of the cell (Wallis, et al., 1983). Furthermore, large C-terminal deletions of histone H2B are lethal, suggesting a vital role for this portion of the histone.

The H4 genes are interchangeable (Mittman, 1984). Studies of histone H4 deletion mutants constructed using oligonucleotide-directed mutagenesis have revealed that much of the amino terminus and a smaller portion of the

carboxyl terminus are not essential (Kayne,et al., 1988). Conversely, sequences in the hydrophobic core region of histone H4 have been shown to be necessary for cell viability (Kayne, et al., 1988). Unlike the case with the H2B deletions, large N-terminal deletions of H4 cause the G2 phase to be irregularly extended and chromatin structure to be altered (Kayne, et al., 1988).

Studies of point mutants have increased understanding of the role of the amino terminus of histone H4 regarding its post-translational modification, acetylation. Four lysines (positions 5, 8, 12, and 16) in histone H4 are known to undergo reversible acetylation which has been associated with the initiation of nuclear functions such as transcription, replication, and chromatin assembly (Megee, et al., 1990). Alteration of these residues was either lethal to the cell or resulted in significant phenotypic changes. Interestingly, this research revealed that no individual lysine residue was vital for cell viability. Rather, the collective effect of loss of multiple lysines proved lethal, perhaps resulting from significant modification of histone-DNA and/or histone-histone associations needed for normal functions (Megee, et al., 1990).

Our Current Model System

The objective of this study is to determine if Saccharomyces cerevisiae (with nonfunctional core histone

genes) can be rescued by a plasmid containing wild-type copies of the four core histone genes. As the first step toward that goal, we have attempted to construct a yeast vector (pRH1, Figure 2), carrying the genes encoding the histones H2A, H2B, H3 and H4, as well as nutritional markers to provide a tracking mechanism for the plasmid throughout this work. Detailed discussion of the intended use of this vector is included in the Appendix.

MATERIALS

Bacterial Strains And Growth Conditions

The bacterial strain DH1 obtained from the laboratory of Dr. Paul Swerdlow, Department of Hematology/Oncology, Medical College of Virginia initially was used for cloning pTS101-2. The bacterial strain JA194 obtained from the laboratory of Dr. Barbara Mittman, Department of Biology, University of Richmond was later used for replacing DH1 in cloning pTS101-2.

Plasmids

Plasmid pTS101-2 (figure 3) was obtained from Dr. Paul Swerdlow, MCV. Plasmid pBAM10/HHF1 (figure 3) was provided by Dr. Barbara Mittman, Univ. of Richmond.

Enzymes

Restriction enzymes, obtained from International Biotechnologies, Inc. (IBI) and Sigma Chemical Company, were used according to instructions provided by the manufacturer.

Calf intestinal alkaline phosphatase and T4 DNA ligase, purchased from Boerhinger Mannheim and IBI, respectively, were used in construction of plasmid pRH1 from pTS101-2 and pBAM10/HHF1.

Electrophoretic Materials

Agarose, obtained from Sigma Chemical Company or IBI, was used in electrophoretic experiments to separate and obtain desired DNA fragments.

Low melting point agarose was purchased from IBI and used according to manufacturer's instructions.

Isobutanol/Hexadecyl Trimethyl Ammonium Bromide ("C-Tab")

'C TAB,' purchased from IBI, was used to extract DNA from low melting point agarose.

METHODS

Bacterial Transformations

Bacterial strains were grown on L-agar plates (1.5% Bacto Agar [Difco], 1.0% Bacto Tryptone [Difco], 0.5% Bacto Yeast Extract [Difco], 1.0% NaCl, pH 7.2) using 100µg of ampicillin [Sigma] for plasmid selection. Overnight cultures were grown in L-broth (1% Bacto Tryptone [Difco], 0.5% Bacto Yeast Extract [Difco], 0.5% NaCl, pH 7.2) using 100µg/ml ampicillin [Sigma]. Bacteria grown on L-agar were maintained at 37°C or in liquid at 37° with shaking.

Bacterial strain DH1 was made competent for transformation by growing a single colony in 10ml of L-broth at 37°C overnight with agitation. A volume of 0.5ml of this culture, used to inoculate 25ml of L-broth, was incubated for 1.5 hours at 37°C with agitation. The culture was centrifuged at 6,000 X g for 10 minutes. Supernatant was discarded. A volume of 12.5ml of a 0.1M $MgCl_2$ solution was added to resuspend the bacterial cells. After recentrifugation, supernatant was decanted and 1.2ml of a 0.1M $CaCl_2$ solution was added. Cells were next placed on ice for 30 minutes to induce competency.

Aliquots of 0.2ml of competent cells were added to 6 different culture tubes for transformation. To tubes 1

through 4, 0.5, 2, 4, and 6µl of pTS101-2 were added. Tube 5 received 2µl of sterile DIH_2O for a negative control and tube 6 received 1µl of pBAM8/hhf1-2 to act as the positive control. Each tube was incubated on ice for 30 minutes then heat shocked at 42 °C for 2 minutes. To each of the tubes, 0.8ml of L-broth was added, and the samples were incubated for 20 minutes in a H_2O bath at 37°. A 0.5ml aliquot was removed from each of the tubes, plated on separate L-Ampicillin (L-Amp) agar plates and incubated overnight at 37 °C. The above procedure was repeated numerous times until it was recognized that DH1 was incapable of undergoing transformation. Consequently, bacterial strain JA194 was used for subsequent transformations.

DNA Minipreparations

Visible colonies resulting from growth of transformed cells were selected from the L-Amp agar plates and used to inoculate 2.0ml of L-Amp broth. The cells were incubated for 5.5 hours at 37 °C. From this culture, 1.5ml aliquots were placed into individual microcentrifuge tubes and centrifuged for 1 minute. The supernatant was decanted and the pellet resuspended in 100µl of an ice cold 50mM glucose, 10mM EDTA and 25mM Tris-Cl solution at pH 8.0. The samples were vortexed and incubated at room temperature for 5 minutes. A 200µl volume of 0.2N NaOH with 1% SDS was added to each of the samples and the tubes gently inverted several times. The samples were stored on ice for 5 minutes

and then 150µl of 3M Na acetate, pH 5.5 was added to each tube. The samples were gently shaken for 10 seconds then again stored on ice for 5 minutes. The tubes were ultracentrifuged for 5 minutes and the supernatant was rapidly transferred to a sterile tube. Phenol extraction was performed on each of the samples by adding an equal volume of phenol/chloroform to each of the tubes. The samples were vortexed then microcentrifuged for 2 minutes. The DNA was washed with 2 volumes of 95% ethanol and microcentrifuged for 5 minutes. The supernatant was decanted and 1ml of 75% EtOH was added to the tube and the pellet was vortexed and microcentrifuged for 5 minutes. The pellet was allowed to dry overnight by inverting the tube. Once dried, the pellet was stored at 4 ^{o}C. The pellet was resuspended in 25µl sterile Tris-EDTA (TE) then 5 and 10µl samples were placed into 2 respective tubes. One sample (experimental) was digested with EcoRI and the other (control) sample was undigested. The two samples were electrophoresed on a 1% agarose gel and run against a lambda Hind III size marker for comparison. Lambda Hind III exhibits seven distinguishable bands upon electoelution of the following kilobase values: 23, 9.9, 6.6, 4.4, 2.2, 1.98, 0.5 (see Fig. 18, for example). These values provide a consistent marking system for comparing experimental digests of (un)known DNA samples to define fragment sizes of such DNA digests.

Large Scale Plasmid Preparations

A 25ml aliquot of L-Amp broth was inoculated with a single transformed bacterial colony containing the pTS101-2 vector. The culture was incubated overnight at 37°C with shaking. Five ml of this overnight culture was placed in 500ml of L-Amp broth (1:100 dilution) and incubated for 3.5 hours at 37°C with shaking. Two ml of a 34mg/ml of chloramphenicol in 95% ethanol was then added to the culture and incubated for another 16 hours at 37°C with shaking. The cells were then centrifuged at 6,000 X g, the supernatant decanted, and the pellets resuspended in 5ml of a 25% sucrose in 50mM Tris, pH 7.5 solution.

Two ml of an ice cold 10mg/ml lysozyme solution was added to the samples. These samples were placed on ice for 5 minutes with intermittent swirling. A 2ml volume of 0.5M EDTA, pH 8.0, was then added and the solution was incubated on ice for another 5 minutes with gentle swirling. Five ml of Lysis mix (0.1% Triton X-100, 62mM EDTA, 50mM Tris, pH 8.0) was added to each of the samples and incubation was allowed to continue for another 20 minutes on ice. The solutions were centrifuged at 12,000 X g for 40 minutes. The supernatant was decanted, the volume measured, and an equal number of grams of cesium chloride were added. A 1/10 volume of 5mg/ml ethidium bromide was added, and the samples were placed in ultracentrifuge tubes and balanced. The tubes were spun at 38,000 X g for 40 hours. Under

ultraviolet light, the lower band DNA in the tube was removed by puncturing the tubes with a 21 gauge needle and extracting the DNA in a 3ml syringe. The DNA was immediately dialyzed in 1 X TE (10mM Tris and 1mM EDTA, pH 8.0) for 1 hour, phenol extracted, then dialyzed overnight with one subsequent TE change during this dialysis.

Plasmid Construction

Partial Digestion Conditions

For pTS101-2 And pBAM10/HHF1

The DNA concentrations for pTS101-2 and pBAM10/HHF1 samples were determined by the following formula:

[DNA] = (Absorbance)(Dilution Factor)(50µg/ml/OD Unit), where 50µg/ml/OD Unit is a constant for this equation. After plasmid concentrations were calculated, DNA digest experiments were performed using 1 unit of enzyme for every µg of DNA present. For the pTS101-2 vector, a partial digest using EcoRI was required to extract the 4.02 kb fragment containing the H2A and H2B genes. A full or complete digest of this plasmid would yield the undesired linearized 12.61 kb fragment, impossible for ligation into the pBAM10/HHF1 vector. Similarly, partial digestion of pBAM10/HHF1 using EcoRI was necessary since a complete digest of this plasmid removes the gene encoding tryptophan, an essential marker for later experiments.

EcoRI was added to plasmid pTS101-2 in a ratio of 1µg plasmid to 1 unit of restriction enzyme and incubated at

37°C for 30 seconds to 30 minutes. Maintaining concentrations of enzyme to DNA on a 1:1 ratio (1 unit/1 µg), partial digests for both plasmids were based entirely upon experimental trial and error. DNA digestion trials were performed by setting up a number of experimental tubes containing the digestion mixtures and testing the optimum time limits which yielded a partial digest of the respective vectors. Partial digestion resulting in the linearization of pBAM10/HHF1 occurred under similar conditions as pTS101-2, with a partial digest requiring an incubation time anywhere from 10 seconds to 70 minutes. After the respective incubations for partial digestion, the mixtures were heat shocked at 65°C for 10 minutes. Samples were taken of the digestion mixtures and run on a 1% agarose gel with a lambda Hind III marker.

DNA Band Isolation And Recovery :

DEAE-Nitrocellulose Paper Technique

When the H2A-H2B 4.02 kb fragment had separated from the pTS101-2 vector, the current was stopped. Similar procedures were followed for the linearized 7.845 kb pBAM10/HHF1 band. Both plasmid bands (the linearized 7.845 kb pBAM10/HHF1 vector and the 4.02 kb insert from pTS101-2) were extracted from the agarose gel using a DEAE nitrocellulose membrane. A piece of the DEAE paper was hydrated using sterile DIH_2O and placed in electrophoresis buffer (A 1:20 dilution of 98.6g Tris base, 54.4 Na

acetate, 7.44g EDTA and 5mg ethidium bromide per litre, pH 7.2). A small incision was placed just ahead and parallel to the band of interest of both vectors and current was applied to electrophorese the DNA onto the nitrocellulose paper. This paper was then placed into a microcentrifuge tube containing 0.5ml of a high salt buffer (10mM Tris pH7.4, 1mM EDTA, and 1.5M NaCl) and incubated at 65°C for 20 minutes with intermittent vortexing. A 1µl tRNA sample from E. Coli [Sigma] and 1.5ml of 95% EtOH were added to the mixture then subjected to an incubation of -70°C for 30 minutes. The sample was microcentrifuged, washed with 75% EtOH, repelleted, lyophilized, reconstituted with 20µl of 10mM TE and then run on an agarose minigel to determine DNA recovery.

Phenol-Extraction Of Agarose Gel Technique

When the H2A-H2B 4.02 kb fragment had separated from the pTS101-2 vector, the current was stopped. Similar procedures were followed for the linearized 7.845 kb pBAM10/HHF1 band. The agarose gel containing the band of interest was excised and placed in a microcentrifuge tube and mashed with a pasteur pipet. One hundred µl of phenol was added and the suspension was then vortexed for 10 minutes and then placed at -70°C for 20 minutes. The sample was microcentrifuged for 15 minutes and the upper aqueous phase was then decanted. Two extractions with equal volumes of phenol were performed and an appropriate volume of 3M

sodium acetate (pH 5.5) was added to make a final concentration of 0.3M. The mixture was ethanol precipitated, washed with 75% EtOH, and lyophilized for approximately 2 hours. The precipitate was reconstituted with 20µl of sterile TE and a sample run on an agarose minigel to verify DNA recovery.

DNA Extraction From Low Melting Agarose Using Isobutanol/Hexadecyl Trimethyl Ammonium Bromide (C-TAB)

In a separatory funnel, 150 ml of N-Butanol were added to an equal volume of distilled water. After 50 inversions, the phases were allowed to separate and placed in separate containers. One gram of C-TAB was then dissolved in 100 ml of the (upper) equilibrated butanol phase. After 50 inversions, the solution was allowed to separate overnight. Each phase was placed in a separate bottle and warmed to 37 ^{o}C.

When the H2A-H2B 4.02 kb fragment had separated from the pTS101-2 vector, the current was stopped. Similar procedures were followed for the linearized 7.845 kb pBAM10/HHF1 band. The agarose gel containing the band of interest was excised and placed in a glass tube and dissolved at 65-70 ^{o}C and the volume was measured. Equal volumes of both pre-warmed aqueous and isobutanol phase were added and the samples were vortexed and allowed to separate at 37 ^{o}C. The upper butanol layer was placed into

another tube. Two further extractions of the aqueous layer were performed using the pre-warmed butanol phase only. The sample was vortexed and allowed to separate at 37°C. The three butanol samples were pooled, removing an aqueous phase that precipitated. One-quarter volume of 0.2M NaCl was added, vortexed, and allowed to separate. The lower aqueous phase was transferred to a new tube. This NaCl extraction was repeated and the aqueous layers were pooled. An equal volume of chloroform was added dropwise to the aqueous pool and the sample was placed on ice to precipitate C-TAB. The upper aqueous phase was removed and 2X volume of 95% ethanol was added. The precipitate was reconstituted with 20µl of sterile TE and a sample run on an agarose minigel to verify DNA recovery.

Electroelution Of Agarose Gel Technique

When the H2A-H2B 4.02 kb fragment had separated from the pTS101-2 vector, the current was stopped and the agarose band containing the desired fragment was excised using a scalpel. Similar procedures were followed in excising the linearized 7.845 kb pBAM10/HHF1 band. The respective bands contained in the agarose sections were then placed in separate dialysis tubes and a sufficient volume of running buffer to fill the dialysis tubing was added to the contents inside each of the dialysis bags. Using plastic clamps, both ends of the separate bags were sealed off and the dialysis bags were placed against a

current of 200 volts for two hours to run the DNA from the agarose gel onto the interior side of the dialysis tubing. The agarose sections were removed from each of the bags, then the bags positioned with the polarity reversed for two minutes to electroelute the DNA sample into the contained running buffer. The contents were then removed and placed into a microcentrifuge tube. A final concentration of 0.3M Na acetate (pH 5.5) was added to the tube followed by the addition of 2X 95% EtOH and the mixture was vortexed and placed at -20 °C overnight. The sample was removed and microcentrifuged for ten minutes and the supernatant was decanted. The remaining contents were lyophilized for several hours and reconstituted with 20µl of TE and vortexed. A 5µl sample was removed and run against a lambda Hind III marker on a 1% minigel to validate that the respective DNA bands were recovered.

Ligation Of The 4.02 kb Fragment Into The 7.845 kb Linearized Plasmid

Plasmid pRH1 was constructed using the 4.02 kb fragment from the partial digestion of the outermost EcoRI sites on pTS101-2 and inserting this fragment into the partially digested, phosphatased linearized pBAM10/HHF1 EcoRI site. Phosphatase acts by removing the 5' phosphate groups from the cut ends of this vector which are necessary for the formation of phosphodiester bonds, thereby inhibiting recircularization of the plasmid (Maniatis, et al., 1985;

1989). The insert from pTS101-2 contains the wild type genes for histone H2A and H2B. The pBAM10/HHF1 vector contains the wild type genes for histones H3 and H4 as well as a gene coding for the amino acid tryptophan that is utilized as a marker.

The ligation of insert to linearized vector was performed using approximately 0.05 pmoles of insert and adding this to 0.02 pmoles of pBAM10/HHF1. This represents a 2.5 fold insert to vector ratio. An amount of 0.5µl of T4 DNA ligase [IBI] with 9.0µl of 10X salt was added to this mixture and incubated for ligation at 10 ^{o}C overnight. The control used contained 0.002 pmoles of pBAM10/HHF1 vector with 0.5µl ligase with 1.0µl 10X salt to 10µl sterile H_2O.

Steps Which Would Have Followed The Successful Construction of pRH1 :

Sporulation and Tetrad Dissection

Progeny resulting from the mating of yeast strains BAM 202 and TSY-157 (figure 10) would be grown on selective media (figures 11 and 12) to promote loss of the pTS101-2 vector. The resulting diploid cell (figure 12) would then be allowed to sporulate. The succeeding tetrads would be individually separated by a technique known as tetrad dissection (figure 13). This procedure would be executed to facilitate crude identification of the desired yeast strain

from nonparental tetrads that result from meiosis.

Plasmid Dependence Test

The plasmid dependence test (figure 14) would rely on the ability of yeast cells which no longer contain the pTS101-2 vector, to grow in tryptophan rich media. The cell populations which are able to grow would contain either functional or nonfunctional histone gene copies yet all would presumably contain pRH1. Further selective growth conditions would confine the possible cell populations to containing all chromosomal copies of the histone genes nonfunctional yet surviving on plasmid borne copies of pRH1.

RESULTS

Transformation : Bacterial Strain JA194 Replaces DH1

The single colony of DH1 proved incapable of transformation by pTS4. Analysis using DH1 with other plasmids (example, pBAM8/hhf1-2) and the respective controls [postive controls using other bacterial strains for transformation (example, JA194) and negative controls (using no vector)] demonstrated that the colony of DH1 was incapable of transformation. Although other bacterial strains were capable of transformation (example, JA194) by the pST-4 vector, the single colony obtained of DH1 was considered to have lost this ability. Consequently, further experiments were conducted using a different bacterial strain, JA194, to provide large scale plasmid preparations. Once the transformation of pTS-4 was verified, large scale plasmid preparations were performed. These large scale plasmid preparations were necessary to generate large amounts of plasmid for future restriction digest investigations. Large plasmid production allows more restriction digests, examined by electroelution. Furthermore, large scale plasmid production facilitates future transformation experiments. Restriction digests and recovery were carried out to obtain sufficient quantity of the 4.02 kb band insert from pTS-4 for future cloning

experiments.

Transformation : Plasmid pTS4 Discovered To Be pTS101-2

Digestion experiments demonstrated a third EcoRI site (which upon complete digestion yielded DNA fragment sizes : 1.76 kb, 2.25 kb, and 8.6 kb) (Figure 17). It was determined that pTS-4 (Figure 15) was plasmid pTS101-2 (Figure 3) which contains a third EcoRI site adjacent to the genes coding for histone H2A and H2B. This finding was very significant to this work for several reasons. The third EcoRI site found in pTS101-2 lies between the genes coding for histone H2A and H2B. Flanking these genes are the other two existing EcoRI sites. The mapping distance between an adjacent EcoRI site to the EcoRI site between the H2A and H2B genes is approximately 2.25 kb. The opposite flanking EcoRI site is approximately 1.76 to 1.77 kb to this central EcoRI site. Thus, the total distance between the two EcoRI sites (which contains the H2A, H2B, and third EcoRI site is approximately 4.02 kb. The mapping distance between these flanking EcoRI sites (not including the 4.02 kb region containing the H2A and H2B genes) is approximately 8.6 kb. Consequently, a complete or full digestion of pTS101-2 would yield a 2.25 kb fragment containing the H2A gene, a 1.76 kb fragment containing the H2B gene, and an 8.6 kb fragment which contains a gene coding for the amino acid uracil and several other genes to be ignored at present. The genes of interest lie in the

4.02 kb fragment, namely the genes coding for histones H2A and H2B. A complete digest would thus disrupt this fragment and would likely inhibit the transfer of the H2A and H2B genes into the receiving pBAM10/HHF1 vector.

pRH1 Construction

Optimum conditions for the partial digestion of pTS101-2 and of pBAM10/HHF1 with EcoRI were necessary to obtain the H2A-H2B 4.02 kb fragment from pTS101-2 (Figure 17) and to linearize the pBAM10/HHF1 plasmid (Figure 18). A ratio of one unit of EcoRI to 1µg pTS101-2 was consistently employed. Similar conditions were met for the partial digestion of pBAM10/HHF1. Maximum yields of the 4.02 kb insert (Figure 19) were found to occur at varying incubation times of the digestion mixture, varying from 30 seconds to 30 minutes. Maximum yields for the linearized 7.845 kb pBAM10/HHF1 vector (Figure 20) varied with incubation times, from 10 seconds to 70 minutes. Digestion methodology for both plasmids consistently followed an established protocol, yet diverged extensively in the rate of reaction time. Large scale partial digests of linearized pBAM10/HHF1 were immediately phosphatased (Figure 21) to prevent recircularization of the plasmid vector DNA.

Several established protocols were employed to isolate and recover the 4.02 kb of pTS101-2 and the linearized full length plasmid (7.845 kb) pBAM10/HHF1 bands separated during electrophoresis. DEAE-nitrocellulose paper technique

did not recover DNA and was abandoned after numerous trials since the DNA could not be successfully recovered. Phenol extraction of the agarose gel did not recover DNA. Low melting agarose using C TAB (or isobutanol/hexadecyl trimethyl ammonium bromide) (Bingham, et al., 1978) did not extract DNA. Electroelution was effective in recovering the H2A-H2B DNA fragment and the linearized pBAM10/HHF1 vector. Both electrophoretic gels displayed the respective DNA bands of the two experimental plasmids.

DISCUSSION

The pivotal step propelling this work was the construction of the plasmid pRH1 (figure 2) from pTS101-2 (figure 3) and pBAM10/HHF1 (figure 4). Without construction of pRH1, subsequent transformation of this newly made vector into the yeast strain BAM 200 was not possible. Nonetheless, this work has produced findings which describe the optimum conditions and techniques that should greatly facilitate future pursuits in the construction of such a described yeast strain.

Identification of pTS101-2

The plasmid originally identified as pTS-4 (figure 15) was determined to be another plasmid pTS101-2 (figure 3). Complete EcoRI digestion of pTS-4 should have yielded a 4.02 kb band containing the genes encoding for histones H2A and H2B, and a 12.6 kb band containing the gene for uracil. A complete digestion of 'pTS-4' with EcoRI resulted in a 2.25 kb fragment (presumably containing the gene for histone H2A), and a 1.76 kb band containing the H2B gene. A third band recovered was 8.6 kb which contained the gene for uracil (Figure 17). Numerous restriction digests were made and each of these provided evidence that there was a third EcoRI site on the plasmid (Figure 17). This third unexpected restriction site was of serious consequence to

the construction of pRH1 (Figure 2) because its location was adjacent to the genes coding for histones H2A and H2B. Consequently it was recognized that the plasmid received was not pTS-4 (Figure 15) but actually pTS101-2 (Figure 3). This conclusion was based on comparisons to published restriction enzyme maps for the plasmids (Schuster, et al., 1986).

Construction of pRH1

The significance of pTS101-2 identification was that a partial digestion of this vector was necessary and essential in order to recover the 4.02 kb band containing both histone H2A and H2B for subsequent insertion into the linearized pBAM10/HHF1 plasmid. An alternative approach considered was based on collecting the separate H2A (2.25 kb) fragments and the H2B (1.76 kb) fragments from numerous large scale (complete) digests of this vector. An immediate problem recognized with this approach was that these two fragments, once ligated for insertion into the pBAM10/HHF1 plasmid, may not be properly oriented for correctly coding for the H2A and H2B histones. In addition, multiple copies of each of the genes could be ligated together prior to insertion into pBAM10/HHF1. A lack of useful restriction enzyme sites in the genes rendered interpretation of these alternative outcomes difficult. Not until much later in this work would the results of this approach be known, hence it was believed that a more conservative approach

involving the full removal of the 4.02 kb fragment containing the proper H2A-H2B orientation should be employed. This strategy would presumably discount any future problems that the pRH1 vector construct would have in coding for these two proteins. A partial digestion for pBAM10/HHF1 (Figure 4) was also of importance since this vector contains two EcoRI sites immediately adjacent to the nutritional marker tryptophan. A partial digestion of pBAM10/HHF1 was therefore necessary to ensure that the gene coding for tryptophan remained in this plasmid. Consequently, once the 4.02 kb insert from pTS101-2 (Figure 3) was ligated into the linearized partial digestion of pBAM10/HHF1, the tryptophan marker would provide a tracking measure for following the pRH1 construct in subsequent steps of this work.

Factors Involved in Partial Restriction Digests

Optimum conditions for the partial digestion for both pTS101-2 and pBAM10/HHF1 were experimentally determined. One unit of enzyme (EcoRI) was used to digest 1µg of plasmid at an incubation temperature of 37 ^{o}C, though partial digestion rates varied considerably for all plasmid preparations. Since the amount of plasmid band isolation after centrifugation may vastly differ among preparations, plasmid preparations were individually analyzed to determine vector concentration. Subsequent partial digestions providing maximum yields of the desired DNA

fragments varied considerably for each plasmid preparation. Reasons which account for such variations are numerous. DNA plasmid concentrations analyzed may have falsely indicated more DNA than was actually present due to contaminants. A more likely reason which accounts for the broad variability in these digestion experiments is the fact that repeated use of the restriction enzyme (EcoRI) and reaction buffer may have significantly degraded over time. Optimum storage temperature for this enzyme is -20 °C, though use during restriction digests caused brief storage times at 0 °C. In addition, different lots of restriction enzymes were used. All of these factors may have individually or in combination, affected the partial digestion of the plasmid preparations. Thus, variations in the optimum rate of partial digestion for pTS101-2 ranged from 30 seconds to 30 minutes. Similarly, optimum partial digestion for pBAM10/HHF1 preparations fluctuated between 10 seconds to 70 minutes.

Future Partial Digestion Experiments

Although reaction rates for both vectors were found to exist within a broad window of time, such information can nonetheless prove beneficial for future endeavors in constructing this and other plasmids. Attempts to quantitatively obtain the best reaction times may nonetheless vary for the reasons previously mentioned. Correct preparation of all solutions is an obvious

prerequisite to obtaining optimum conditions. In this case constant attention to minute details of personal technique are also important to obtain high DNA yields and optimum purification. It cannot be overly emphasized that enzyme and buffer storage conditions be maintained per manufacturing instructions. Furthermore, the least deviation from the recommended storage conditions while conducting digestion experiments is best for maintaining optimum enzyme activity.

Recognizing the fact that there may be a variety of factors which can affect partial digestions, any amounts of DNA fragments desired should be isolated from the gel and stored for future work. This applies to small and large scale partial digestion experiments so that DNA isolation can continually be maximized as much as possible.

DNA Recovery

The electroelution method was successful in recovering the H2A-H2B DNA fragment and should aid future investigations in this research. This particular technique was found to be less cumbersome than the previously mentioned protocols for band extraction and DNA recovery. Precisely why the DNA bands from the respective digestions of pTS101-2 and pBAM10/HHF1 were not recoverable, using either the DEAE-nitrocellulose, agarose gel phenol extraction, or 'C-TAB,'is not well understood. DNA recovery using these techniques was so low that it was impossible to

view following electrophoresis. The predominant reason for this low recovery may be that the concentration of material was so low that it was not precipitating well.

Future Avenues and Rationale For This Research

Most of the current information regarding histone-histone interactions in the nucleosome have been obtained by *in vitro* methods. The benefit of this work would be in providing a system that allows an *in vivo* analysis of core histone interactions. Once this yeast strain is constructed, known mutant histone genes would be used in order to study the effects of combinations of various mutant histones. For example, such a yeast cell system may facilitate rapid analysis of the phenotypic effects various frameshift or point mutations at the carboxyl or N-terminal end of histone H3 will have on the cell. Of particular interest, H2A-H2B viable mutants constructed by Ryowski, et al., could be combined with H3-H4 viable mutants constructed by Mittman, et al., for immediate analysis.

Of equal significance to studying regions within the histone genes deemed important for cellular viability, regions involved in transcription, replication, and chromatin assembly may be better understood. Since such work focuses on eukaryotic organisms, information may be obtained regarding how eukaryotic cells function and are regulated. This system would undoubtedly provide a significant vehicle for future investigations in cellular

research.

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Figure 1. The Organization of the Histone Genes in the Yeast *Saccharomyces cerevisiae*.

There are two copies each of the histone genes H2A, H2B, H3, and H4. These are found to occur as unlinked gene sets (H2A-H2B) and (H3-H4). Note the restriction enzyme sites associated with the gene copies. (R=*Eco*RI, H=*Hind* III).

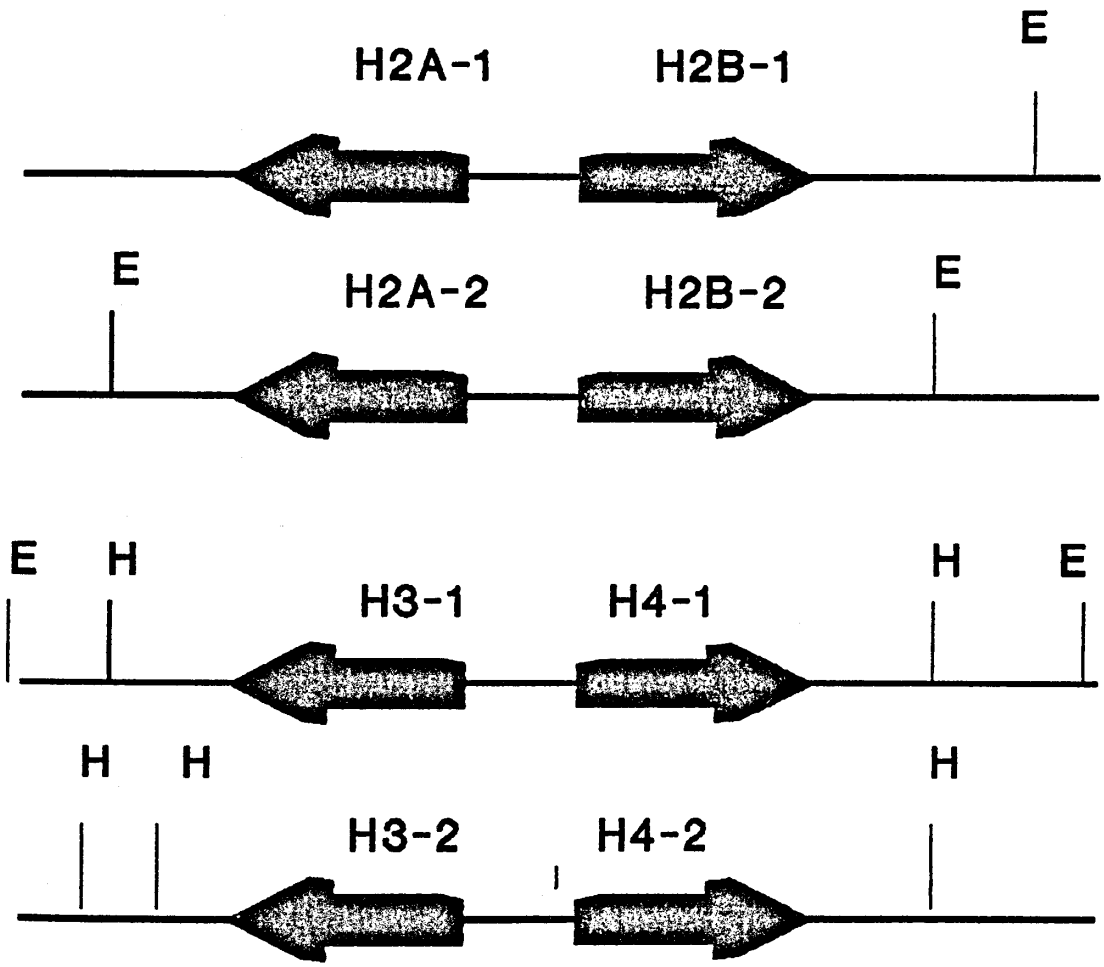

E
H2A-1
H2B-1
E
E
H2A-2
H2B-2
E
E
H
H
E
H3-1
H4-1
H
H
H
H3-2
H4-2

Figure 2. Plasmid Construct pRH1.

This 11.87 kb plasmid contains the 4.02 kb H2A-H2B fragment from pTS101-2 that has been inserted into the pBAM10/HHF1 vector.

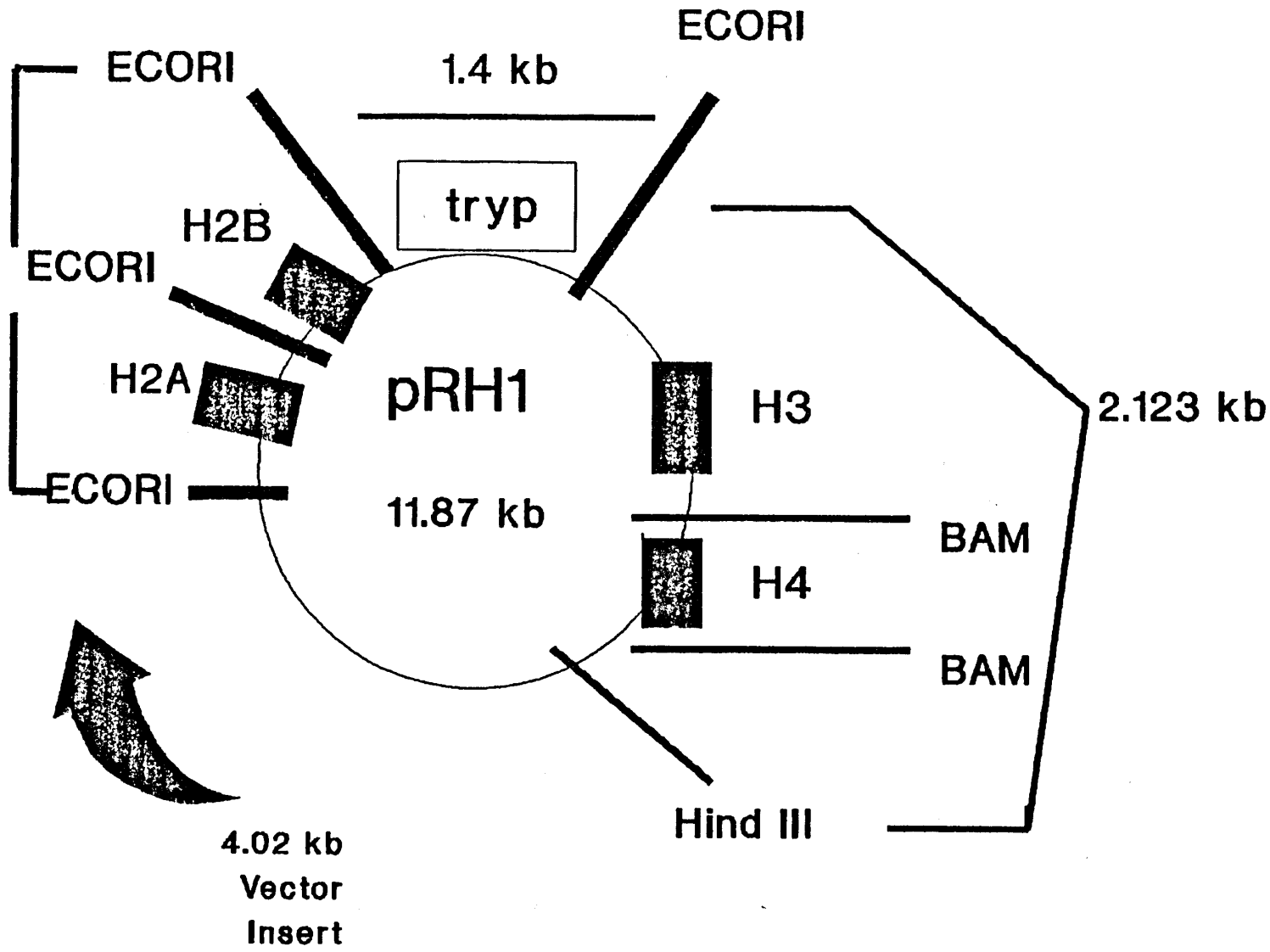

ECORI
1.4 kb
ECORI
tryp
H2B
ECORI
H2A
pRH1
H3
2.123 kb
ECORI
11.87 kb
BAM
H4
BAM
Hind III
4.02 kb
Vector
Insert

Figure 3. Plasmid pTS101-2.

Full and partial digestion of pTS101-2 with EcoRI verified the three EcoRI sites. The 4.02 kb insert was obtained only after precise conditions were discovered ([substrate] : [enzyme] / Time-37^{o}C).

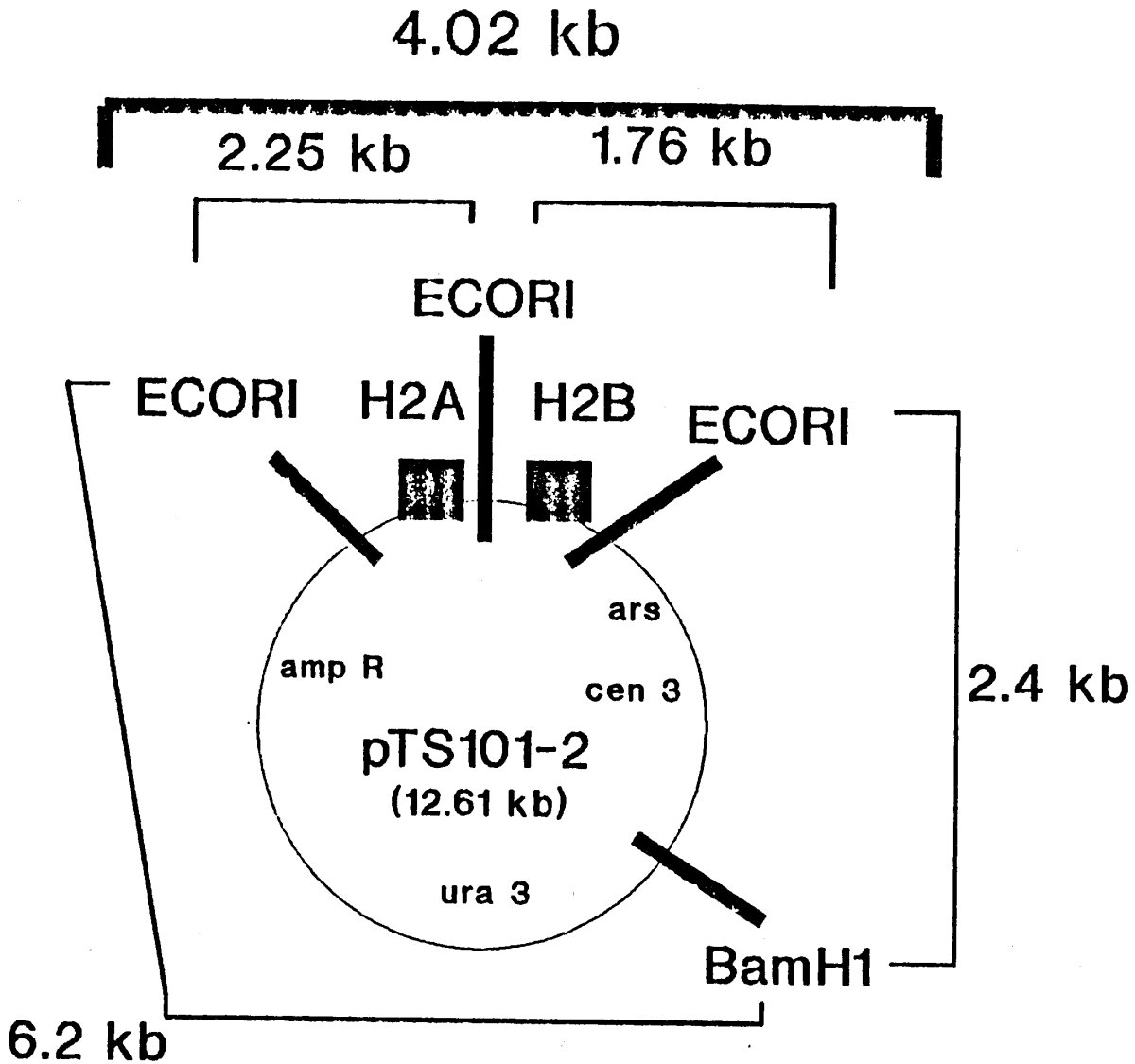

4.02 kb
2.25 kb
1.76 kb
ECORI
ECORI
H2A
H2B
ECORI
ars
amp R
cen 3
pTS101-2
(12.61 kb)
ura 3
2.4 kb
BamH1
6.2 kb

Figure 4. Plasmid pBAM10/HHF1.

This 7.845 kb vector was partially digested with EcoRI and phosphatased for linearization. The tryptophan marker would be utilized as a tracking device for subsequent experiments. This plasmid contains wild type genes for histones H3 and H4 and once ligated with the 4.02 kb fragment from pTS101-2, will contain the wild type genes for histones H2A and H2B.

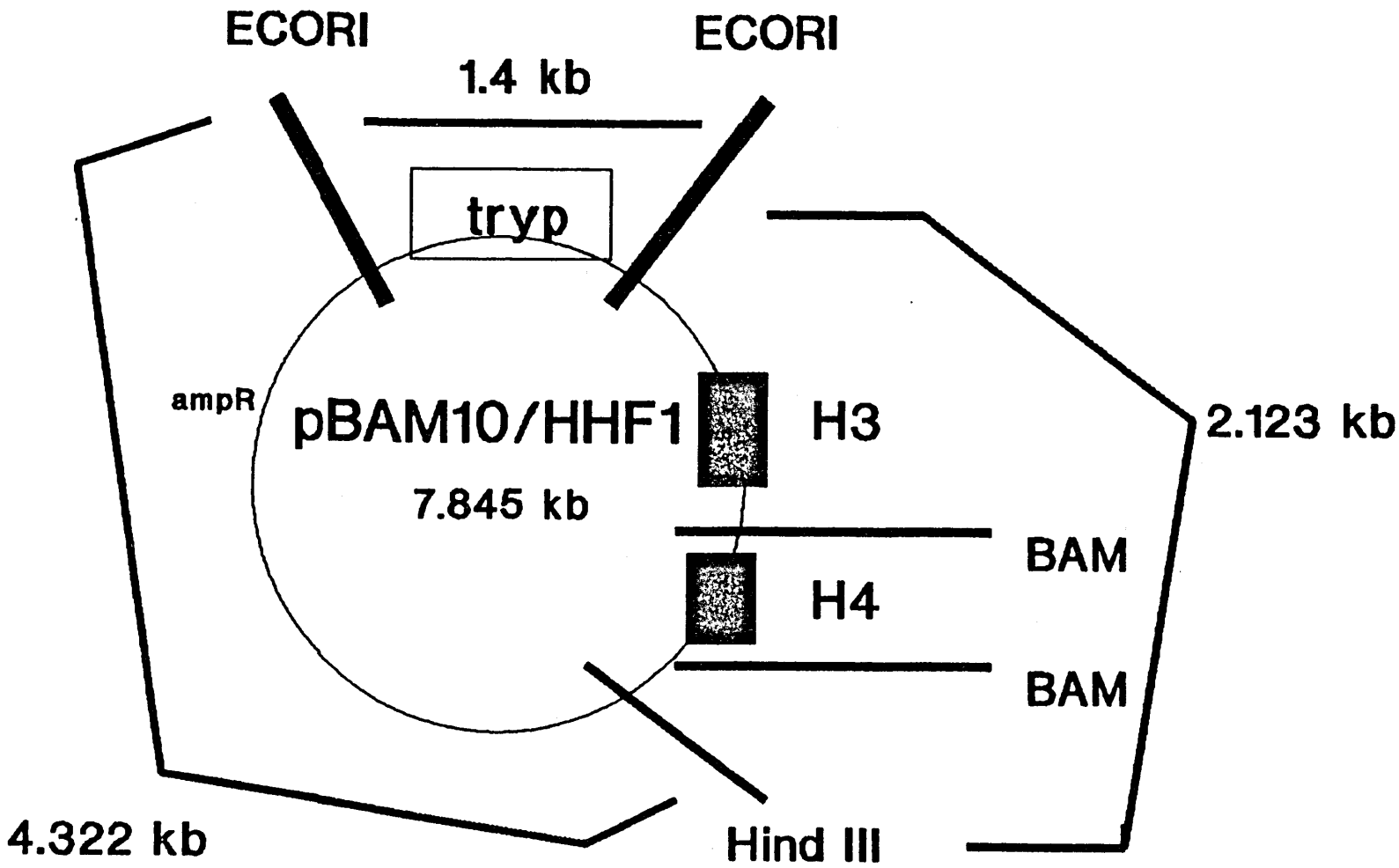

ECORI
ECORI
1.4 kb
tryp
ampR
pBAM10/HHF1
7.845 kb
H3
H4
BAM
BAM
2.123 kb
4.322 kb
Hind III

Figure 5. Transformation of pRH1 into BAM 200.

Transformation of newly constructed pRH1 into the haploid yeast strain BAM 200. BAM 200 contains H3 and H4 deletion mutations and ura 3-52 and tryp 1-289 mutations and survives on plasmid borne copies of H3-1 and H4-1 (pBAM8/476) which also contains a gene for uracil, correcting the cellular defect.

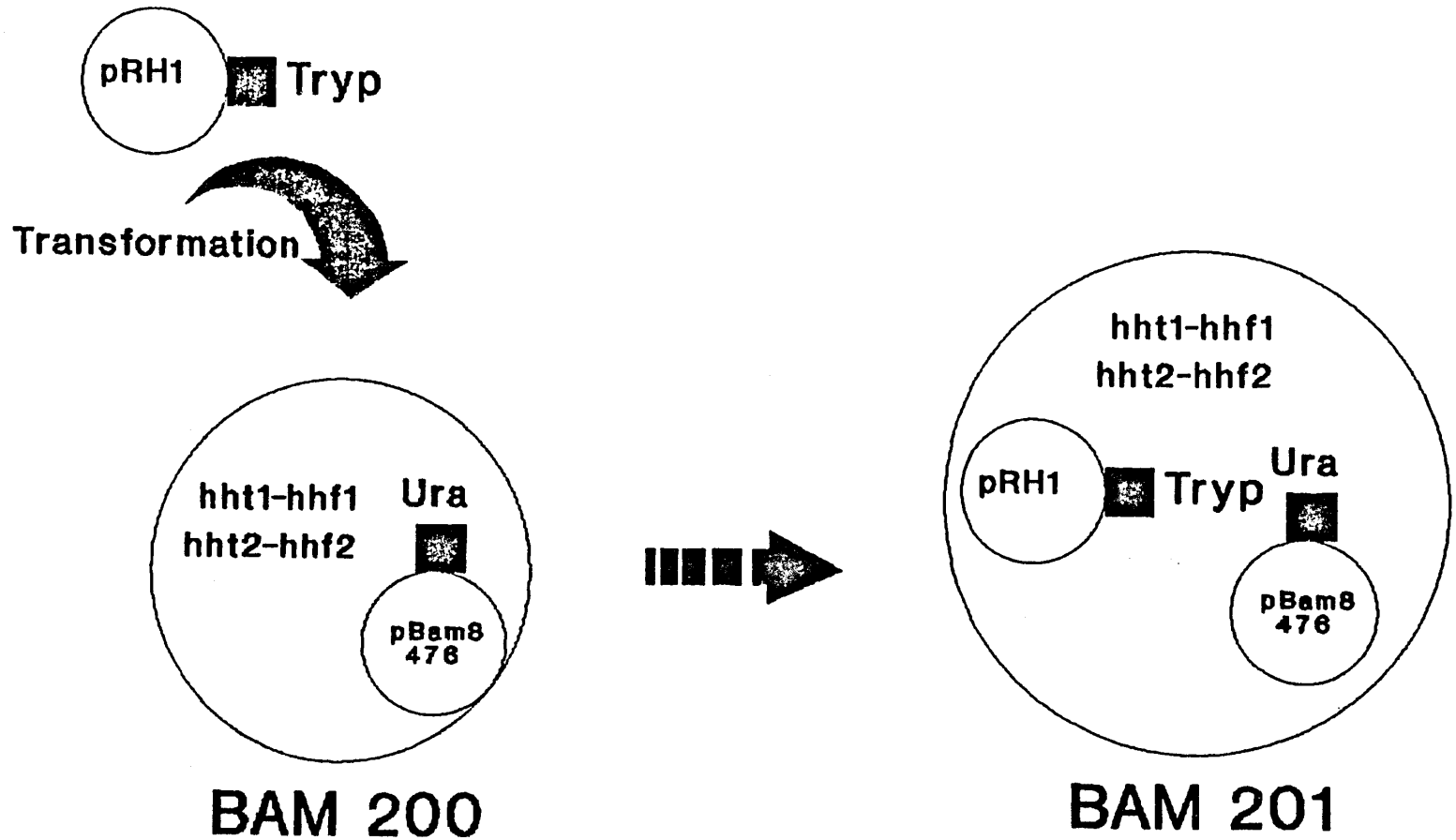
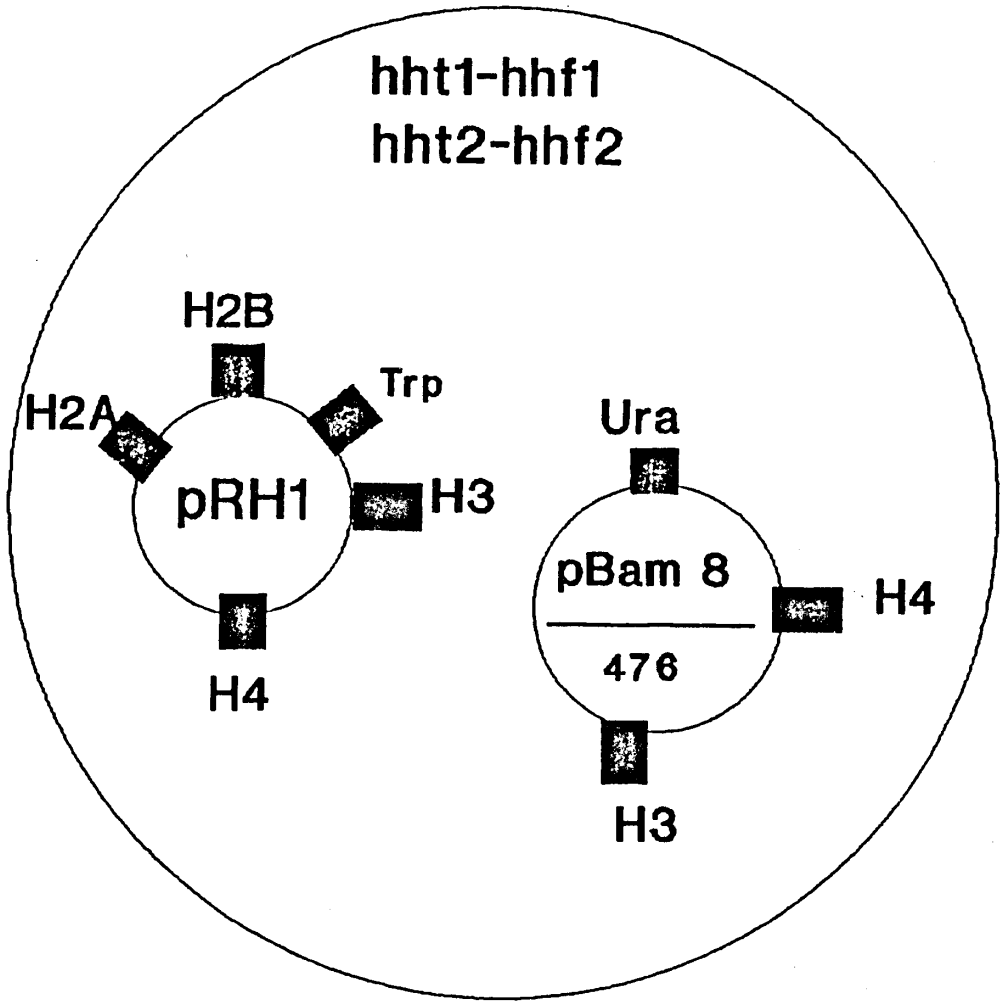

pRH1
Tryp
Transformation
hht1-hhf1
hht2-hhf2
Ura
pBam8
476
BAM 200
hht1-hhf1
hht2-hhf2
pRH1
Tryp
Ura
pBam8
476
BAM 201

Figure 6. Yeast Strain BAM 201.

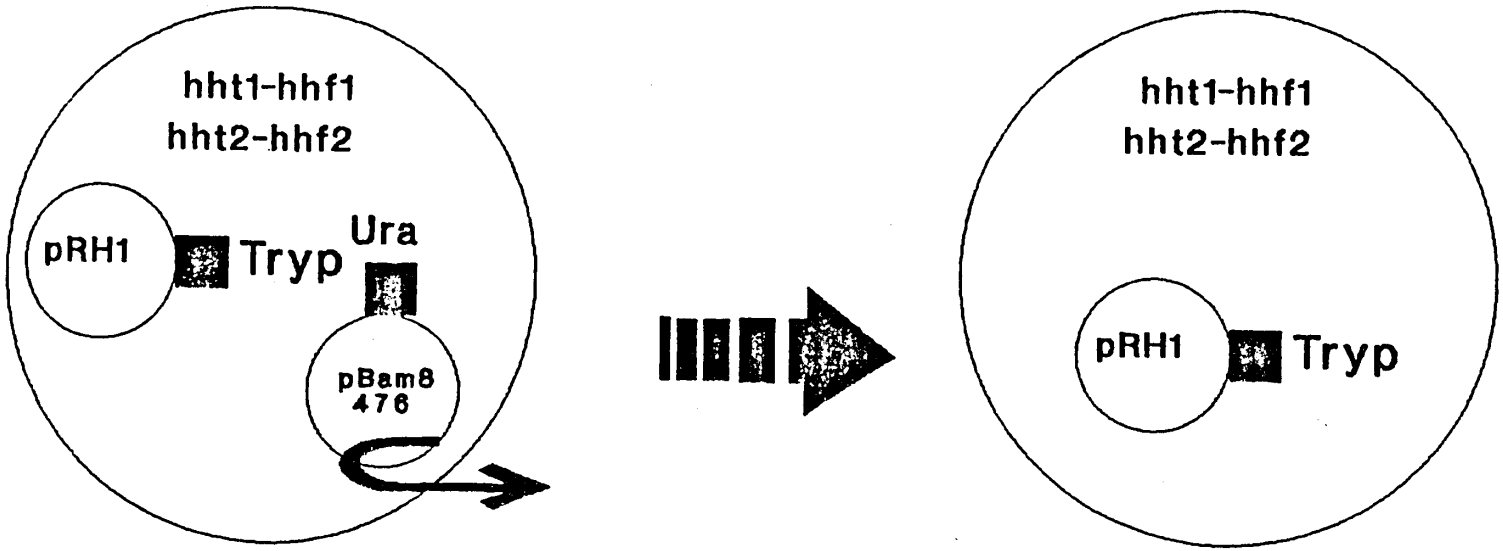
BAM 201 is essentially yeast strain BAM 200 with the addition of the transformed plasmid pRH1. BAM 200 contains H3 and H4 deletion mutations and ura 3-52 and tryp 1-289 mutations and survives on plasmid pBAM8/476 containing the wild type copies of the H3 and H4 genes. BAM 201 will be grown on uracil+/tryptophan- (ura+/tryp-) media to promote loss of pBAM8/476 so that the newly produced strain, BAM 202, survives on pRH1 exclusively.



Bam 201

Figure 7. BAM 201 and BAM 202.

BAM 201 is the resulting strain from the transformation of the pRH1 construct into the yeast strain BAM 200. BAM 201 is then grown on ura+/tryp- media to promote loss of pBAM8/476, resulting in the newly produced strain, BAM 202. This yeast strain survives on pRH1 exclusively.



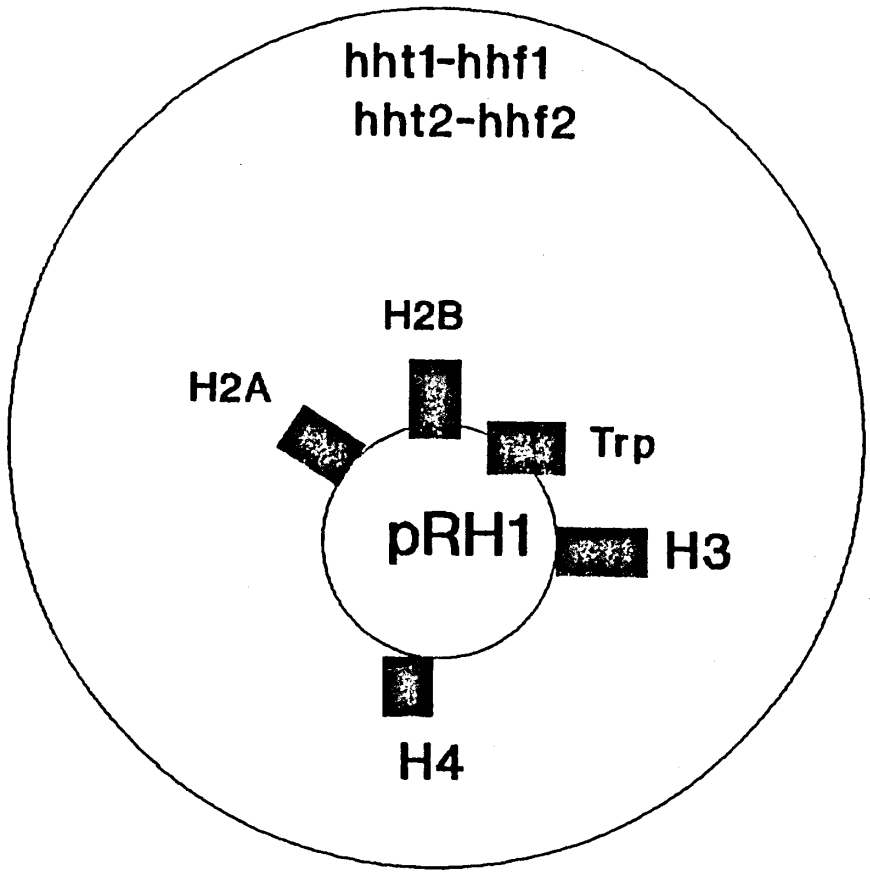
BAM 201

BAM 202

Grow BAM 201 on media containing uracil but lacking in tryptophan to selectively promote loss of pBAM8/476

Figure 8. BAM 202.

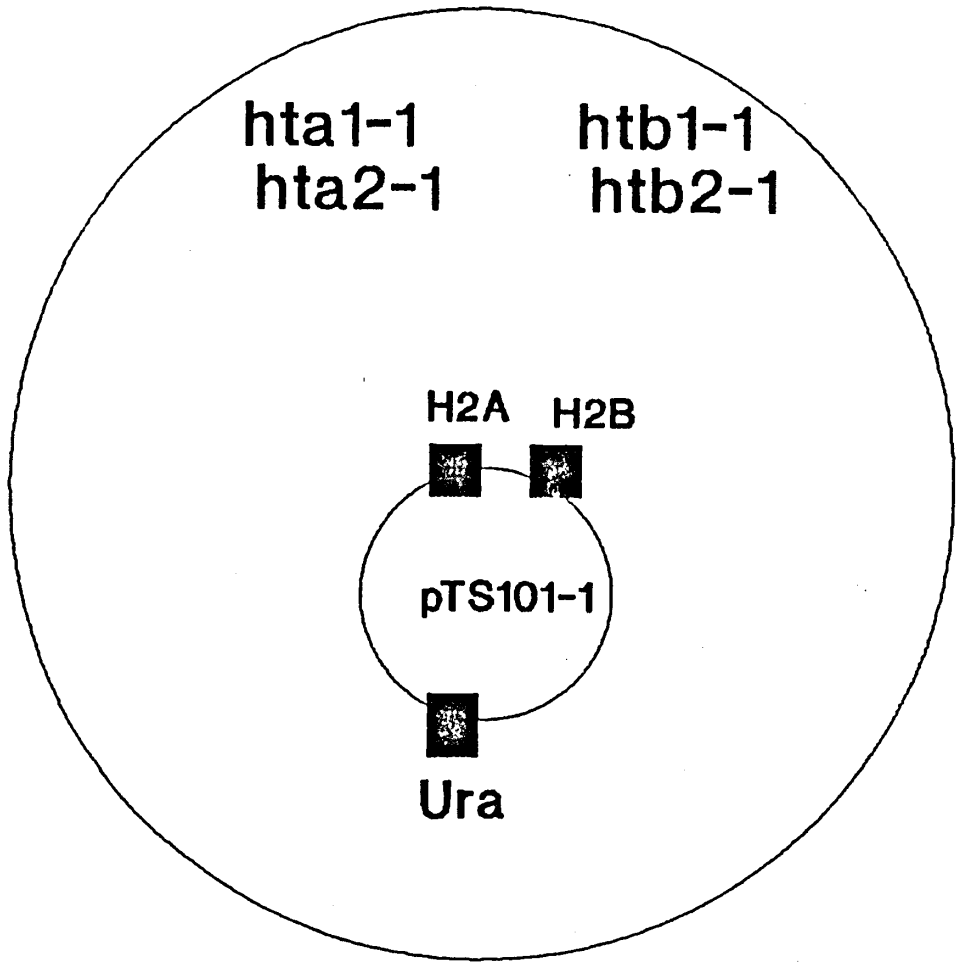
Newly constructed yeast strain BAM 202 contains H3 and H4 deletion mutations and ura 3-52 and tryp 1-289 mutations and survives on plasmid borne copies of H3-1 and H4-1 (pRH1) which also contains a tryptophan gene correcting for the cellular defect.



BAM 202

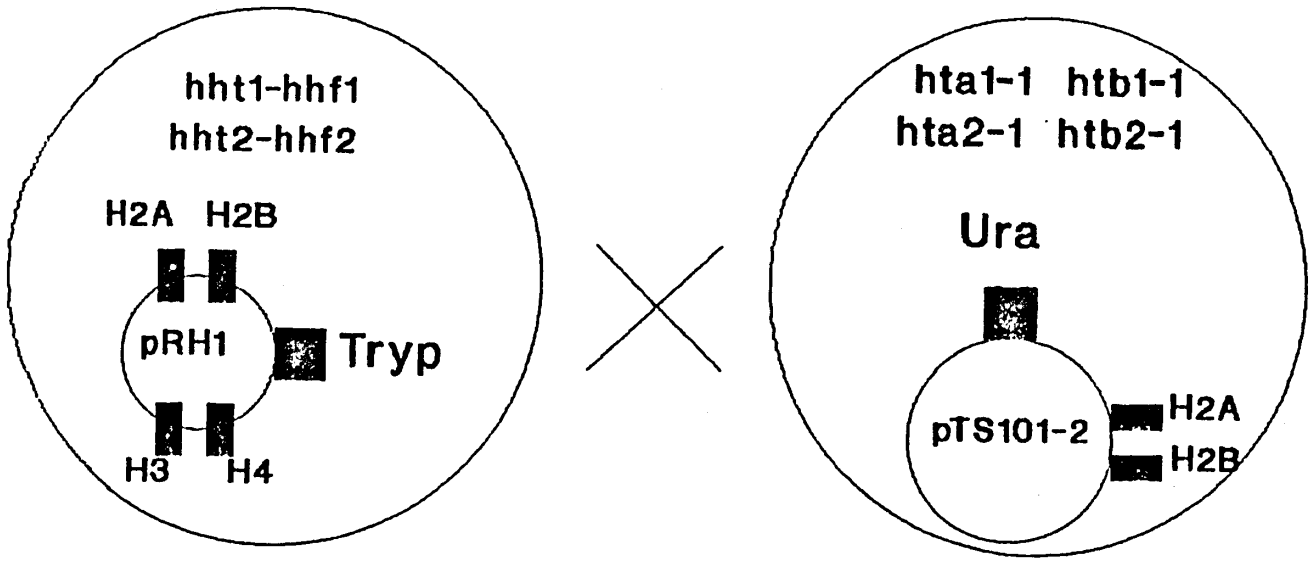
Figure 9. Yeast Strain TSY-157.

Newly produced BAM 202 would be crossed with TSY-157 which contains frameshift mutations of both copies of H2A and H2B but survives on pTS101-2. The resulting diploid cell would be grown on ura+/tryp- media to selectively lose pTS101-2.



TSY-157

Figure 10. Mating of BAM 202 with Yeast Strain TSY-157.

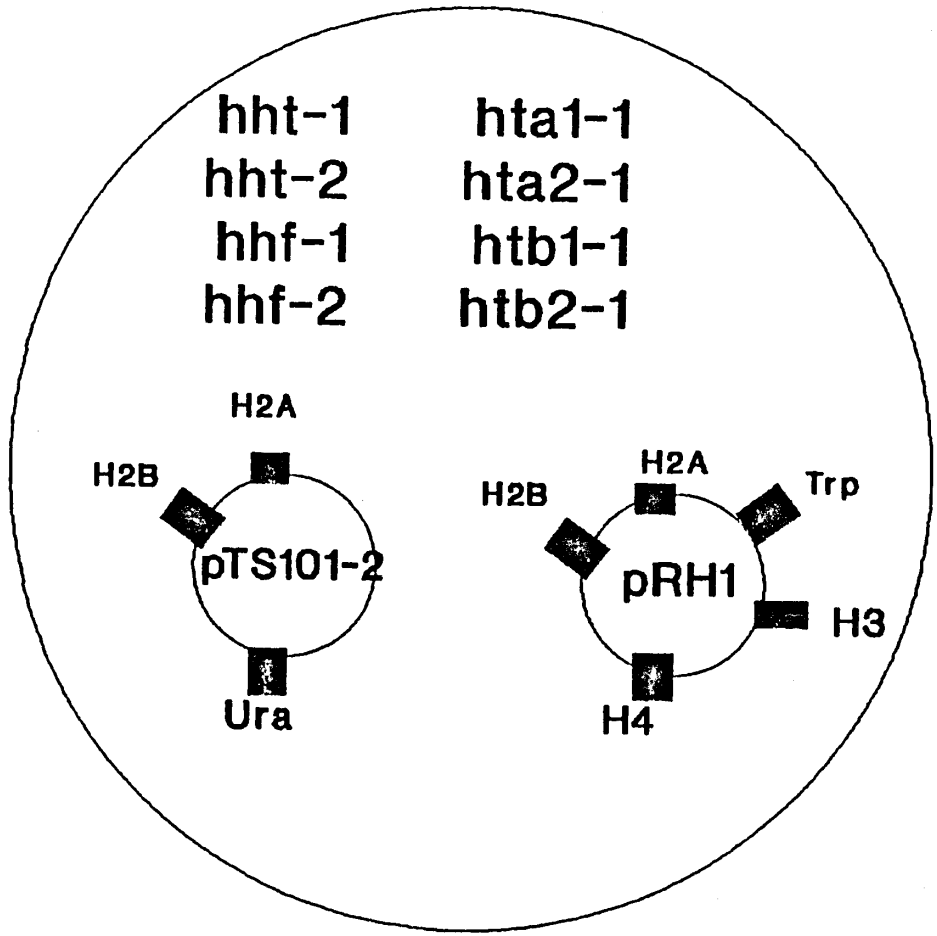


BAM 202

TSY-157

Figure 11. The Diploid Cell 1.

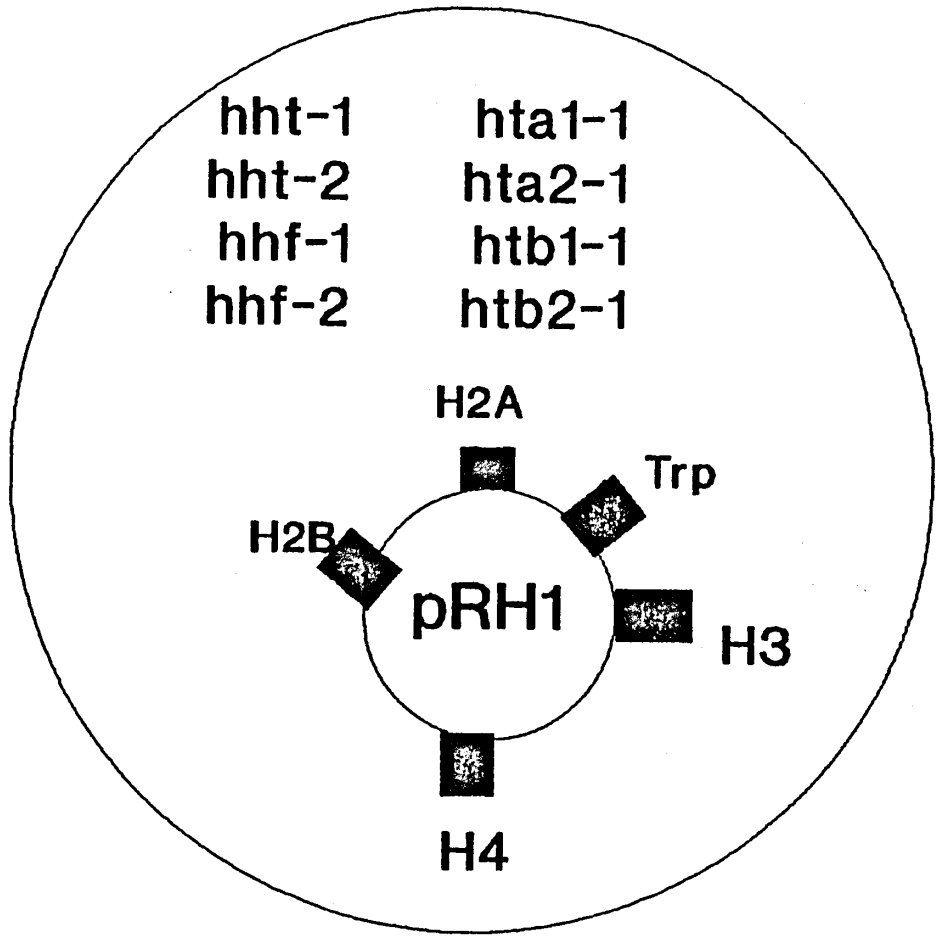
The diploid cell results from the cross between BAM 202 and the yeast strain TSY-157. This diploid cell 1 would be grown on ura+/tryp- media to selectively lose the pTS101-2 vector.


hht-1
hht-2
hhf-1
hhf-2
hta1-1
hta2-1
htb1-1
htb2-1
H2A
H2B
pTS101-2
Ura
H2A
H2B
Trp
pRH1
H3
H4

Diploid 1

Figure 12. Diploid Cell 2.

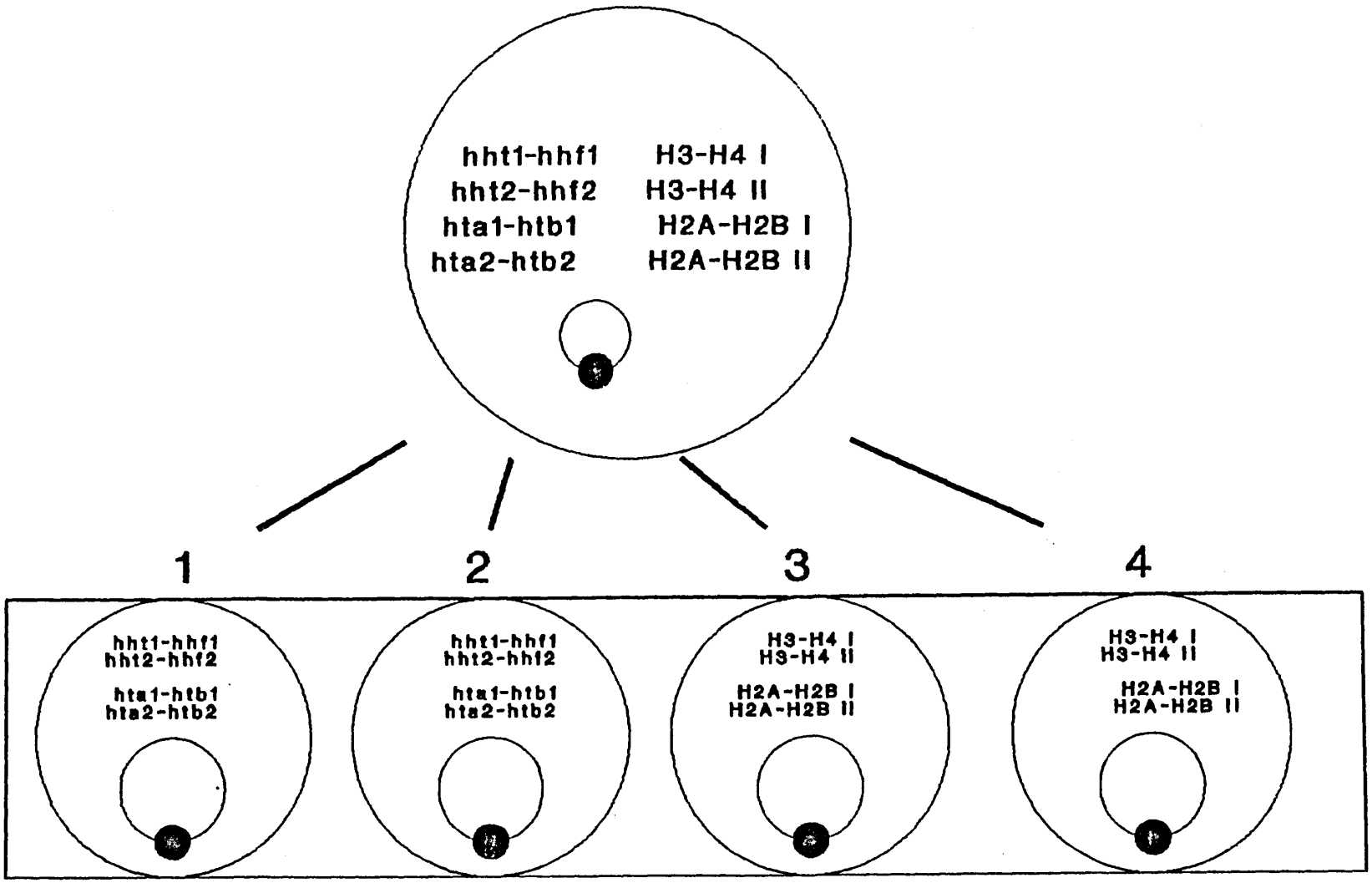
Diploid cell 2 is the result of growing diploid cell 1 (Figure 11) on ura+/tryp- media to selectively promote loss of the pTS101-2 vector.


hht-1
hht-2
hhf-1
hhf-2
hta1-1
hta2-1
htb1-1
htb2-1
H2A
Trp
H2B
pRH1
H3
H4

Diploid 2

Figure 13. Sporulation of Diploid and Tetrad Dissection.

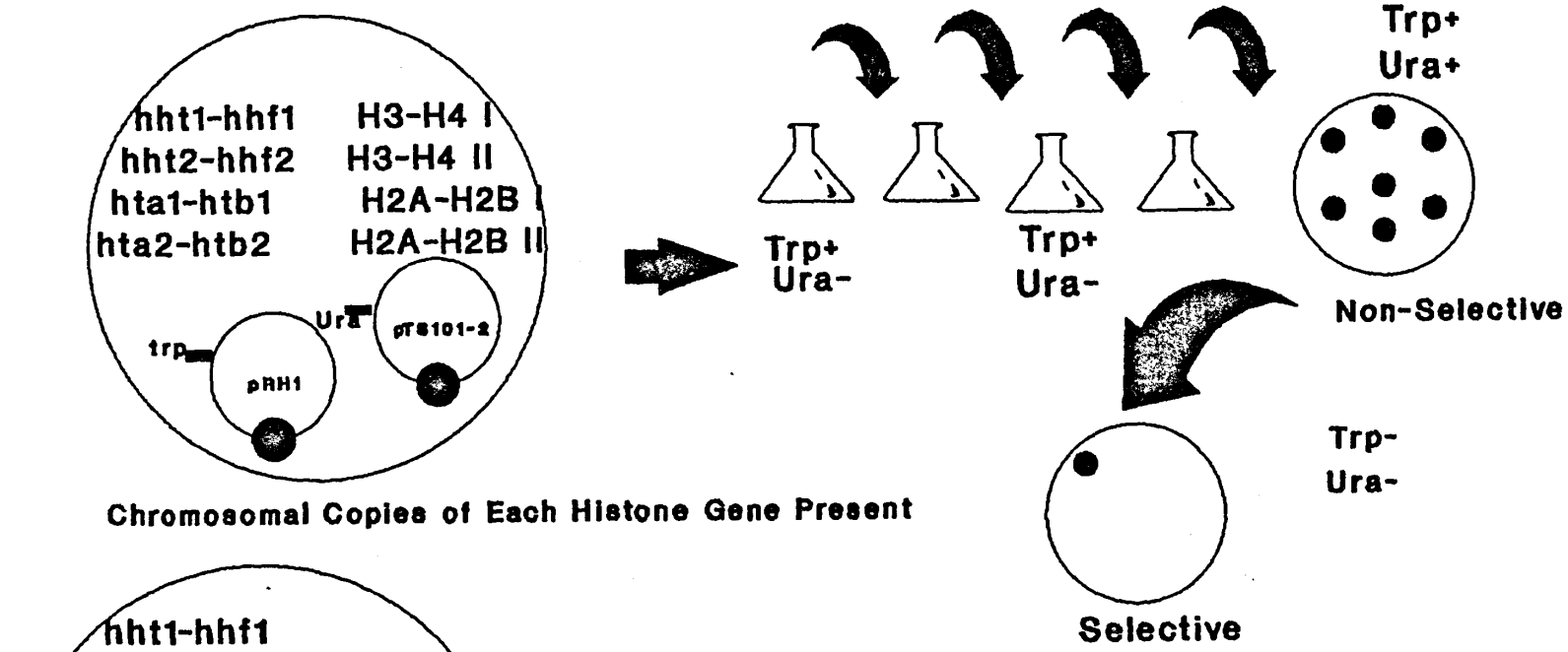
--- Represents pRH1



Sporulation of Diploid and Dissection of Tetrads

Figure 14. Plasmid Dependence Test and Southern Blot Analysis.

After sporulation and tetrad dissection, a plasmid dependence test would be performed to identify that the cell carries nonfunctional chromosomal copies of the histones and is rescued by pRH1. A Southern blot analysis would then be performed so as to confirm the identity of this yeast strain construct.


hht1-hhf1
hht2-hhf2
hta1-htb1
hta2-htb2
H3-H4 I
H3-H4 II
H2A-H2B I
H2A-H2B II
Ura
pTS101-2
trp
pRH1
Chromosomal Copies of Each Histone Gene Present
Trp+
Ura-
Trp+
Ura-
Trp+
Ura+
Non-Selective
Trp-
Ura-
Selective

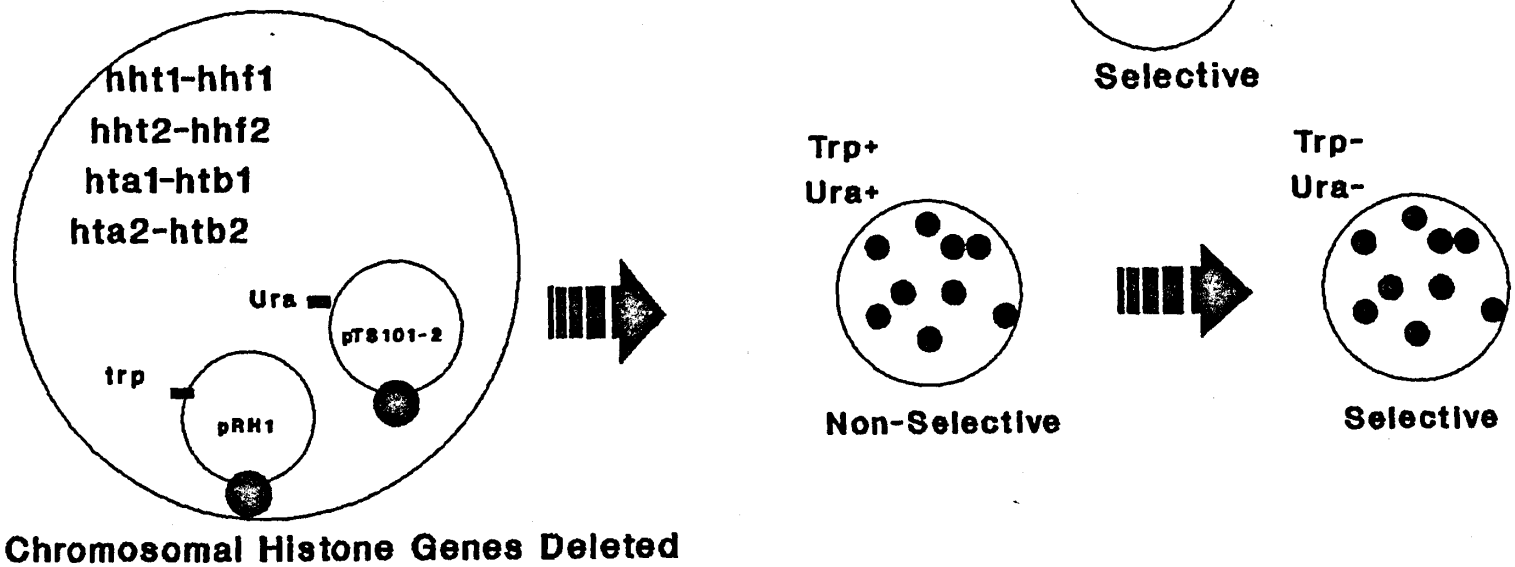

hht1-hhf1
hht2-hhf2
hta1-htb1
hta2-htb2
Ura
pTS101-2
trp
pRH1
Chromosomal Histone Genes Deleted
Trp+
Ura+
Non-Selective
Trp-
Ura-
Selective

Figure 15. Plasmid pTS-4.

The vector donated by Dr. P. Swerdlow, MCV, was originally believed to be pTS-4. Numerous restriction digests confirmed that there was a third Eco RI site within the 4.02 kb fragment, thus indicating that the plasmid was actually pTS101-2. Eco RI digest of pTS-4 would have allowed quicker obtainment of the 4.02 kb insert to place into pBAM10/HHF1 to ultimately form pRH1.

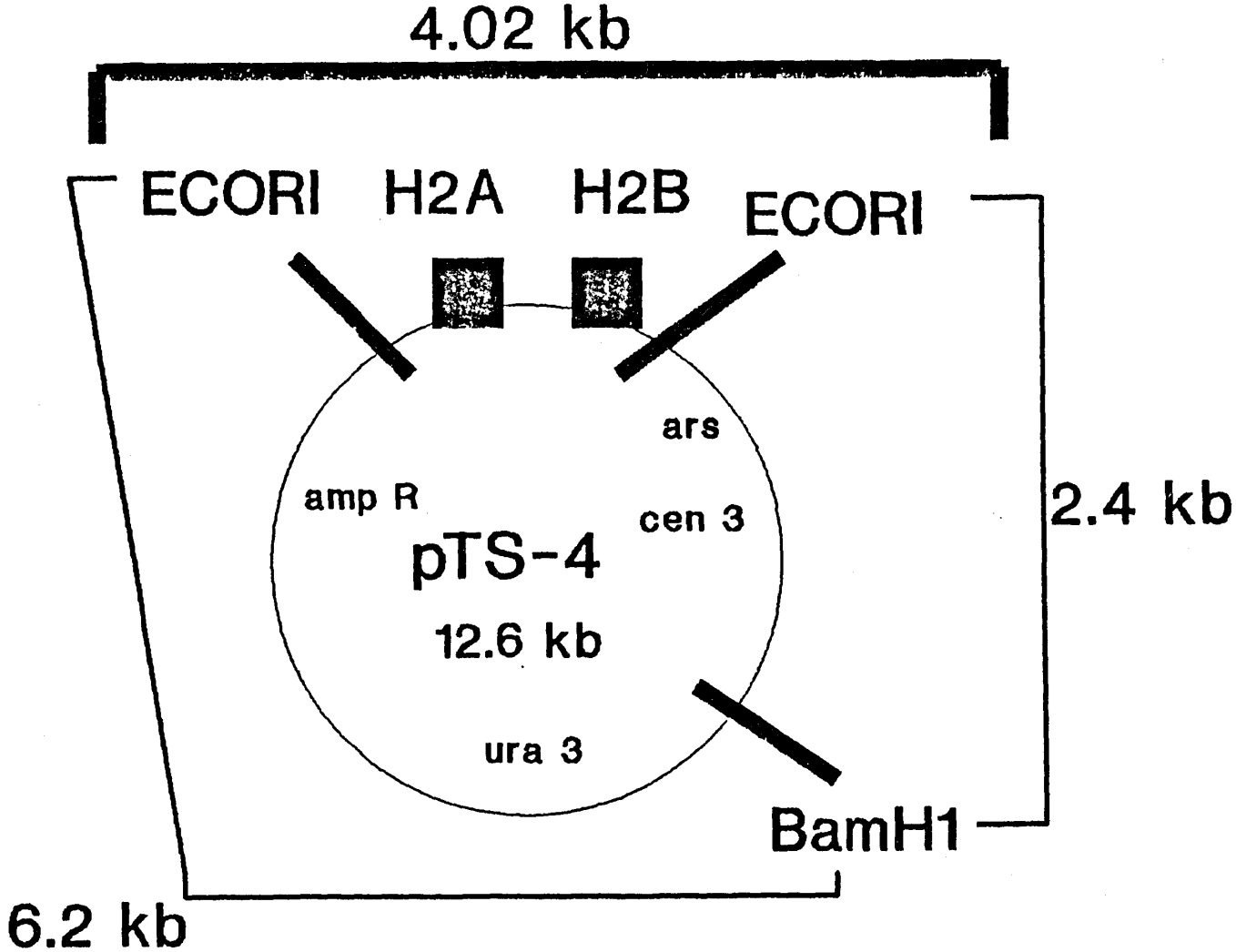
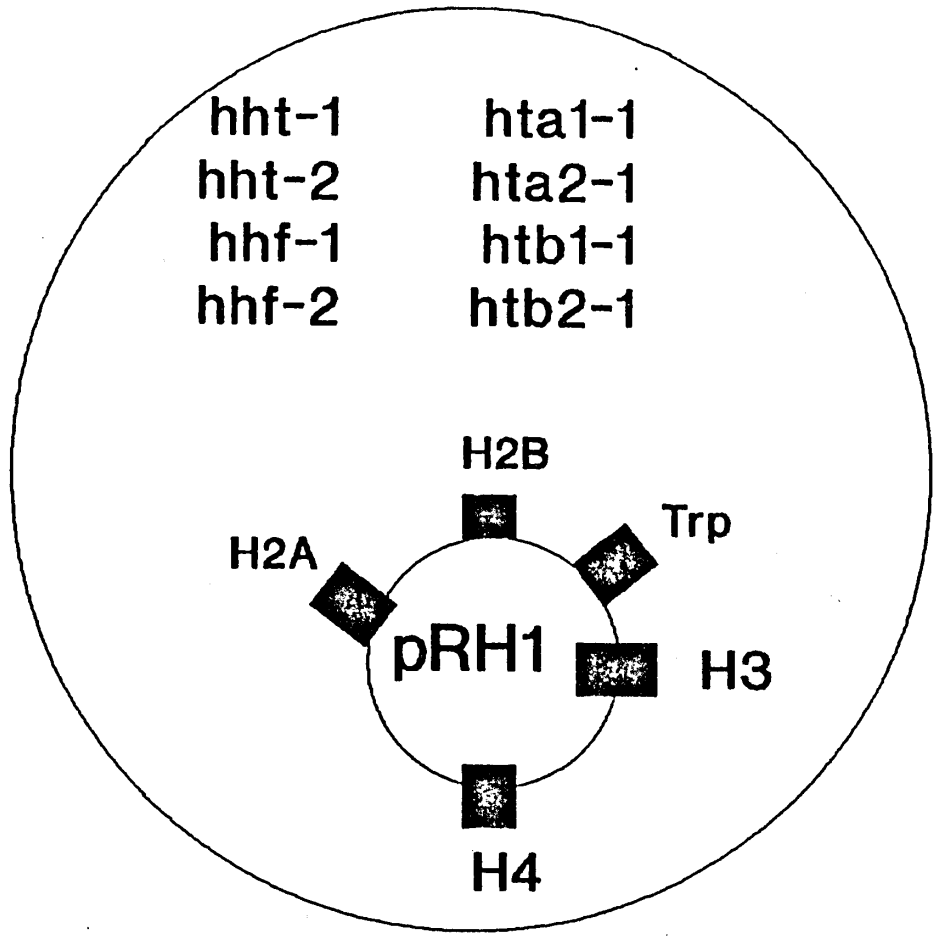

4.02 kb
ECORI
H2A
H2B
ECORI
ars
amp R
cen 3
pTS-4
12.6 kb
ura 3
2.4 kb
BamH1
6.2 kb

Figure 16. Yeast Strain BAM 203.

New yeast strain construct, BAM 203, would contain all chromosomal copies of the histone genes nonfunctional and would be rescued by plasmid borne copies of pRH1.


hht-1
hht-2
hhf-1
hhf-2
hta1-1
hta2-1
htb1-1
htb2-1
H2B
Trp
H2A
pRH1
H3
H4

BAM 203

Figure 17. Electrophoretic Gel Showing Eco RI digests of pTS-4.

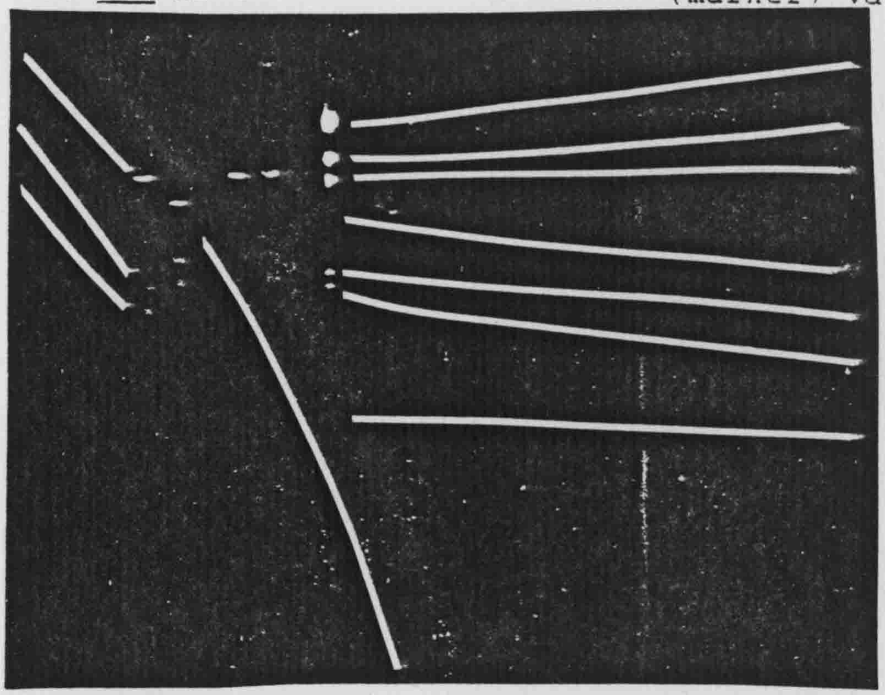
pTS-4 was originally believed to be the vector until numerous restriction digests showed that there were three Eco RI sites on this plasmid. These digests along with digests using BAM HI confirmed that pTS-4 was in fact pTS101-2. When pTS101-2 is fully digested with Eco RI, three bands are seen (as in the photograph) with the following values : 2.25 kb, 1.76 kb, and 8.6 kb. A partial digest of this vector was essential to obtain the 4.02 kb insert containing the H2A and H2B genes. The BAM HI site is located in this 8.6 kb fragment and yields a 6.2 kb fragment plus a 2.4 kb fragment. (Note: Hind III is used as a marker in these digestion experiments).

pTS101-2 complete digest with Eco RI :

8.6

2.25

1.76



lambda Hind III (marker) Values :

23

9.9

6.6

4.4

2.2

1.98

0.5

pTS101-2 complete digest with Eco RI and Bam HI : 6.2, 2.4, 2.25, 1.76

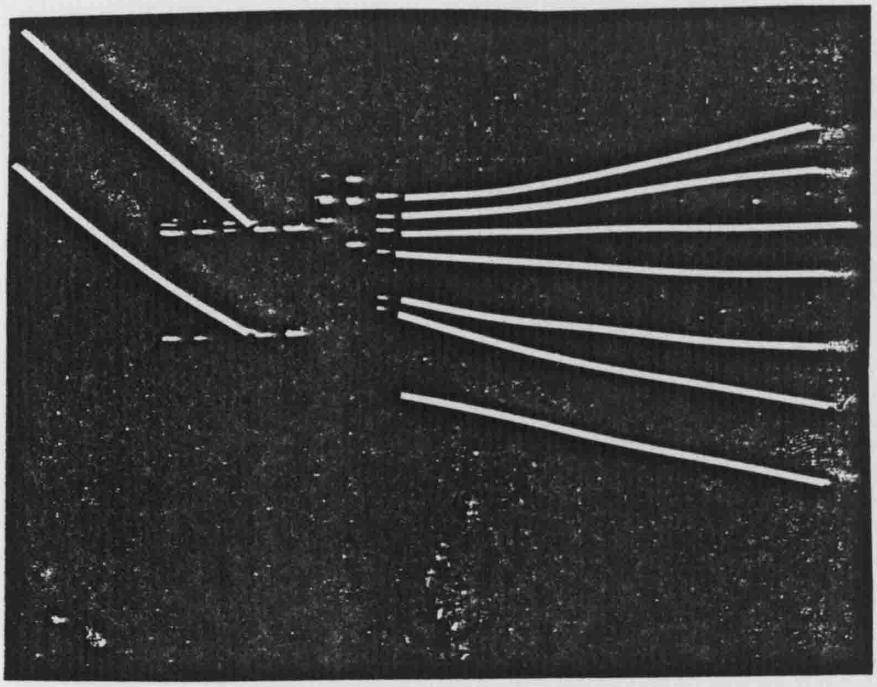
Figure 18. Electrophoretic Gel Showing Eco RI digests of pBAM10/HHF1.

pBAM10/HHF1 when completely digested with Eco RI yields two distinct fragments; a 1.4 kb fragment containing the gene coding for tryptophan, and a 6.445 kb band which contains histones H3 and H4. A partial digestion of pBAM10/HHF1 was essential so that the 1.4 kb fragment containing the tryptophan gene was retained to use for subsequent experiments. (Note: Hind III is used as a marker in these digestion experiments).

pBAM10/HHF1 complete digest with Eco RI :

6.445

1.4



lambda Hind III (marker) Values

23

9.9

6.6

4.4

2.2

1.98

0.5

Figure 19. Partial Digest of pTS101-2 with Eco RI. The band of interest in this photograph is the 4.02 kb fragment from pTS101-2 containing the H2A-H2B genes.

pTS101-2 partial

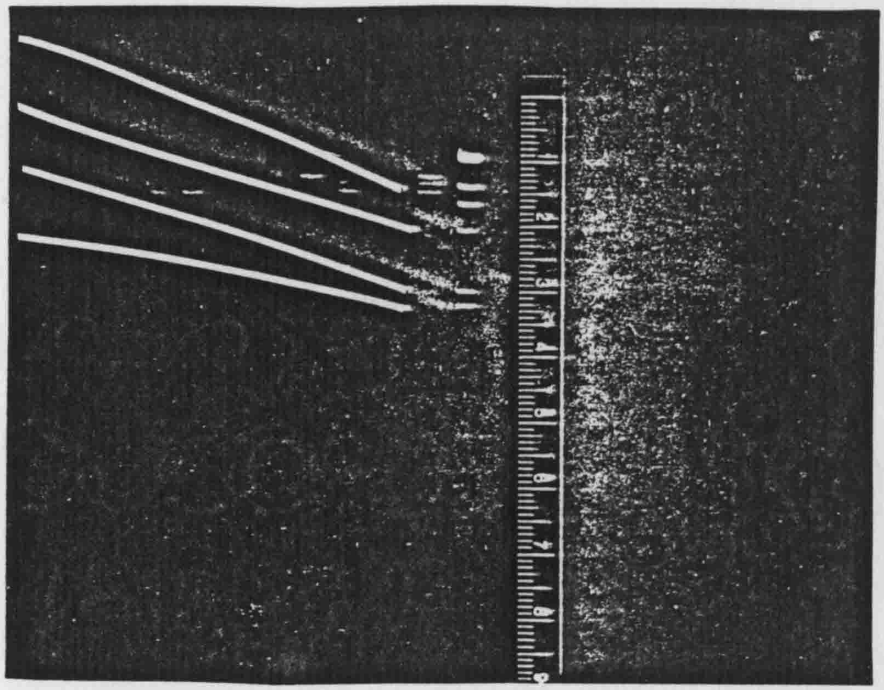
digest with Eco RI :

8.6

4.02

2.25

1.76



lambda Hind III

(marker) Values :

23

9.9

6.6

4.4

2.2

1.98

0.5

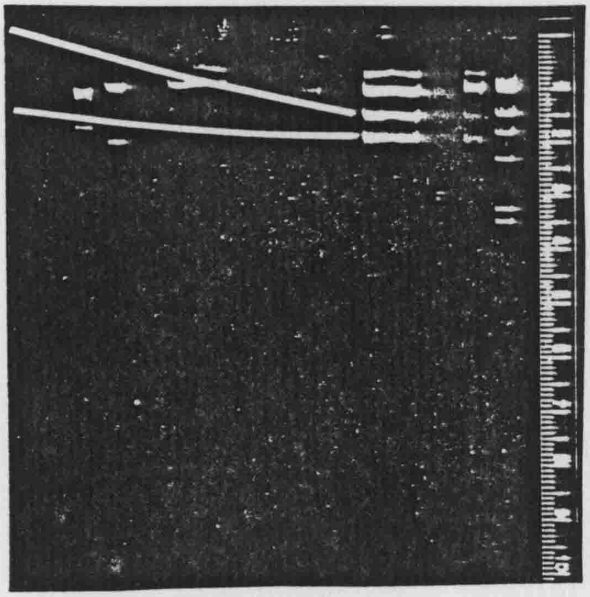
Figure 20. Partial Digest of pBAM10/HHF1 with Eco RI. The band of interest in this photograph is the 7.845 kb linearized vector containing histones H3 and H4 and the gene coding for tryptophan.

pBAM10/HHF1 partial

digest with Eco RI :

7.845 (closed circular)

7.845 (linear)



lambda Hind III

(marker) Values

23

9.9

6.6

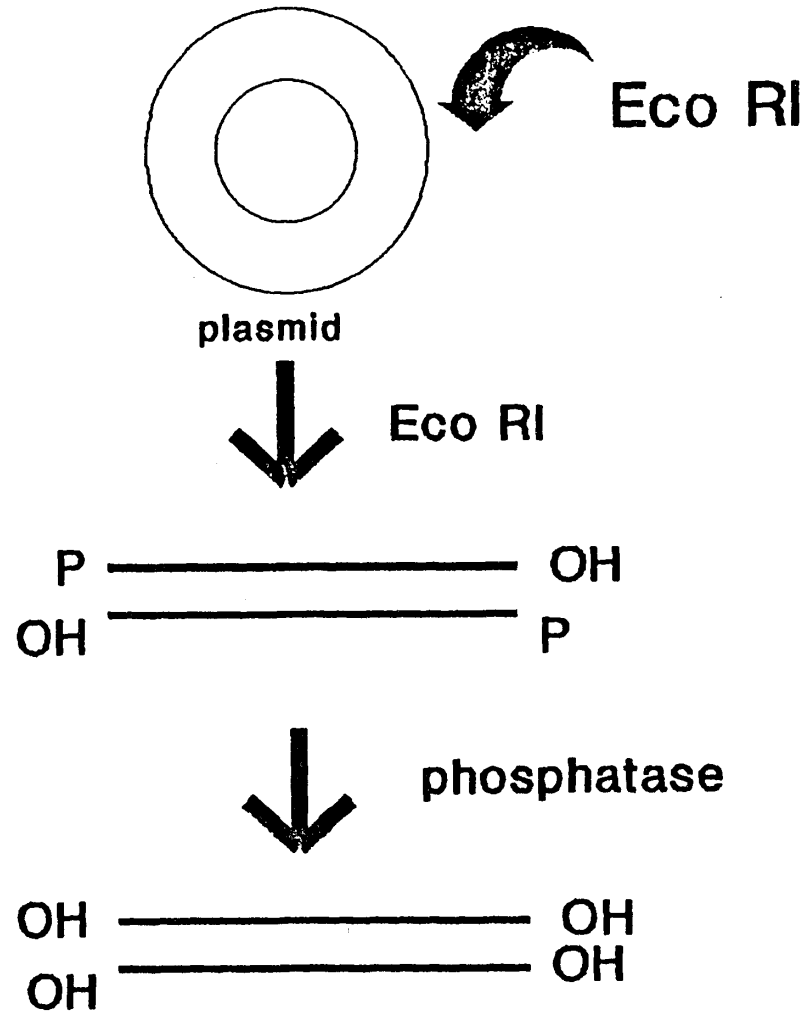
4.4

2.2

1.93

0.5

Figure 21. Diagram Representing the Action of Phosphatase. Phosphatase was used to prevent the recircularization of pBAM10/HHF1 so that the 4.02 kb vector insert from pTS101-2 could be ligated into this linearized plasmid to form pRH1.



Plasmid cannot recircularize

(Appendix)

INTRODUCTION

Historical Perspective On Histones

Histones were initially described in 1884 by Albrecht Kossel from an acid-extractable peptone-like isolate of goose erythrocyte nuclei. Kossel postulated that this component was bound to the DNA in chromatin (Wu, et al., 1987). Initially, various histone classes were defined on the basis of gel mobilities and solubility characteristics. Further elucidation of histones came from electron microscopy studies showing the repetitive structure of chromatin. These repeating sections were defined as nucleosomes, composed of approximately 200 base pairs of DNA coiled with a complex of histones.

There are two components of chromatin, the nucleosome core particle and a linker region which joins these particles (Isenberg, 1979). Chromatin digested with micrococcal nuclease yields a 206 Kd core particle. This particle consists of two each of the protein histones H2A, H2B, H3, and H4 (Richmond, et al., 1984). There are approximately 146 base pairs of DNA coiled around the nucleosome core particle and outside of this central octamer are 20 base pairs of linker region DNA associated with the histone protein H1 (Laskey and Earnshaw, 1980). Interestingly, histone H1 has not been found in yeast

(Matsumoto and Yanagida, 1985). Though the nucleosome core particles are distinctly distributed along the DNA at consistent intervals, the length of the linker DNA has been found to vary among different cell types (Laskey and Earnshaw, 1980). The mechanism which determines the length of this linker DNA between these particles is presently unknown (Laskey and Earnshaw, 1980; Laskey, et al., 1977; Nelson, et al., 1979).

Histones And The Nucleosome

Histones are eukaryotic proteins, however, histone-like proteins have been observed in prokaryotes which also bind to DNA (Rouviere-Yaniv, et al., 1975). During the 1960's, Tonini and Rozjin were the first to characterize yeast histones as basic proteins extracted from yeast chromatin (Smith, 1984). Although histone H1 has never been observed in yeast, yeast core histones are similar to that of higher eukaryotic organisms (Smith, 1984).

Histones are described as a group of small, basic proteins which bind to DNA in chromatin. Sequencing studies have demonstrated that all histones are abundant in basic amino acids and contain highly conserved amino acid sequences (Isenberg, 1979). Only the histone H3 has been found to contain cysteine and only one or two of these residues have been found, depending on the organism (McGhee and Felsenfeld, 1980).

Studies have demonstrated that each of the core particle histones have two specific regions. A globular, hydrophobic region containing a significant amount of α-helix which has been found to be involved with histone-histone and histone-DNA interactions (Kayne, et al., 1988; McGhee and Felsenfeld, 1980) and a second region containing a hydrophilic amino-terminal tail which has a large number of positively charged amino acids (Kayne, et al., 1988). Studies involving trypsin digestion of intact nucleosomes, antibody binding and nuclear magnetic resonance place the basic amino terminus of each histone outside of the inner domain of the nucleosome core particle (Wallis, et al., 1983). Investigations have revealed that the C-terminal portion of histones comprise two thirds of the molecule (McGhee and Felsenfeld, 1980).

Numerous studies have demonstrated that the nucleosome is 5.5nm high and 11nm in diameter (McGhee, et al., 1980). It is believed that the nucleosome is the underlying unit of assembly in the higher order structure of chromatin (Finch, et al., 1977). Investigations on chromatin from chicken erythrocytes using 2M NaCl (pH 7.5) have demonstrated that the histone complex is formed by the organization of two H2A-H2B dimers and one $(H3\text{-}H4)_2$ tetramer (McGhee, and Felsenfeld, 1980; Wu, et al., 1987). Approximately 1.8 turns of a left-handed DNA superhelix are wrapped around the $(H2A\text{-}H2B\text{-}H3\text{-}H4)_2$ histone complex

(Kornberg, et al., 1974; Richmond, et al., 1984). X-ray scattering experiments show that the DNA of the core particle is in the B form (Finch et al., 1980). The DNA duplex is in the right-handed (B) helical form (Finch et al., 1980). Interestingly, as the DNA wraps around the nucleosome, it is found to form a higher order structure as it takes the path of a left-handed helix (Richmond, et al., 1984).

Studies using beef kidney have shown that in the DNA itself, there are 16 helical turns of the left-handed superhelix transversing the nucleosome. Furthermore, there are 10.4 base pairs of DNA which compose each of these helical turns. Consequently this results in 166.4 base pairs abiding in the approximate 1.8 turns of this left-handed DNA pathway (Richmond, et al., 1984; Lilley, et al., 1979).

Crosslinking studies using histone octamers from rat liver have demonstrated that if the DNA superhelix were to be unfolded, the actual linear arrangement of the histones along the DNA would be: H2A1-H2B1-H41-H31-H42-H2B2-H2A2 (Klug, et al., 1984). The core tetramer [$(H3)_2(H4)_2$] has been examined extensively and found to be arginine rich. Histones H3 and H4 have been found to provide nucleosome-like properties on DNA without histones H2A and H2B (Boulikas, et al., 1980), therefore it is presumed that this tetramer is critical in determining the overall

nucleosome structure (Boulikas, et al., 1980; Klug, et al., 1980).

All histones undergo post-translational modifications which has promoted speculation that temporary changes in the histones may have the ability to regulate gene expression. In 1964 it was revealed that histones undergo methylation and acetylation. Two years later, histone phosphorylation was discovered (Allfrey, et al., 1964; Murray, 1966; Kleinsmith et al., 1966). These histone modifications and their effects are currently being studied in several laboratories (Megee, et al., 1990; Schuster, et al., 1986).

Histone-DNA Interactions

Nucleosomal structures have been noted to be absent from highly active gene regions though it is believed that histones are nonetheless present. Stedman and Stedman postulated in 1951 that histones may act as nonspecific gene each repressors whereby their removal would result in gene activation (Lilley and Pardon, 1979). These observations may be due to the fact that on active genes, the transition from nucleosomal structures to uniform fibers is caused by the unfolding of the polynucleosome structures rather than histone absence (Igo-Kemenes, et al., 1982).

Whereas most eukaryotic gene products are made throughout the cell cycle, histone proteins are restricted

to synthesis during S phase, just prior to DNA synthesis and chromosome replication (Maxson, et al., 1983). Studies indicate that if DNA replication is constrained, so is the rate of histone synthesis in a proportionate manner. Thus, DNA and histone synthesis are thought to be connected, implying that histone gene expression are linked with DNA replication (Maxson, et al., 1983).

The most specific information regarding histone-DNA interactions has most recently been made by x-ray crystallography. A 7Å resolution composite of the nucleosome core particle depicts the DNA with possible kinking near regions of physical contact with the histone proteins H3 and H4 (Richmond, et al., 1984). The following year a 3.3Å resolution structure of the core particle was obtained which differed significantly from the Richmond model, displaying the octamer with twofold symmetry (Burlingame, et al., 1985; Wu, et al., 1987). Both models are currently being debated, however the Richmond model is more widely accepted (Klug, et al., 1985).

Histone-Histone Interactions

Due to the advances in DNA sequencing techniques, the deduced amino acid sequence of histones from many species is known (Igo-Kemenes, et al., 1982). It has been observed that most changes are restricted to amino acid substitutions within the hydrophilic amino terminal region (Urban et al., 1979). In yeast Saccharomyces cerevisiae

there exist eight genes per haploid genome which code for the four core histones. There are four pairs for these eight genes which contain either one each of the H2A and H2B gene or one each of the H3 and H4 gene (Smith, 1984) (figure 1). It is well documented from genomic restriction map analysis, genomic blot hybridization, and tetrad analysis mapping that the H2A-H2B pairs are unlinked to either of the H3-H4 gene pairs (Smith, 1984).

Each of the primary histone fractions are composed of isoprotein species or variants (Wu, et al., 1987). Comparing the amino acid sequence of histones from different species appears to indicate that most evolutionary modifications consist of conservative amino acid substitutions situated within the hydrophilic regions which interact with DNA (Urban, et al., 1979). Interestingly, the genes for yeast histones H3 and H4 code for identical H3 and H4 proteins whereas the two H2B genes code for two different H2B variants as is the case for the two H2A genes which code for two different H2A variants (Smith, 1984). Variants of the same histone class are distinguished by changes in the central portion of the hydrophobic area of the histone which is seen to be the region involved in histone-histone interactions (Urban, et al., 1979). Histone H2B contains more evolutionary divergence than histone H3 or H4 but the H2A histone has diverged the most through evolution (Wu, et al., 1987).

There are 135 amino acids comprising the yeast histone H3 protein and 102 amino acids which form the yeast H4 protein (Smith, 1984). Studies have shown that there are 15 amino acid differences between the yeast sequence of histone H3 and the calf thymus histone H3 protein, whereas the yeast H4 protein contains only 8 amino acid differences compared to the H4 protein in the calf thymus (Smith, 1984).

Initial studies concerning histone-histone interactions were performed *in vitro* through physicochemical analyses on the pea plant, *Tetrahymena*, calf thymus and yeast histones (D'Anna and Isenberg, 1973; 1974). Further *in vitro* analyses performed showed similar results which represent three distinct levels of interaction between histone pairs : H2A-H2B, H2B-H4 and H3-H4 form strong pairing while H2A-H3 forms slightly weaker bonding and H2A-H4 and H2B-H3 showed the weakest strength of association (Wu, et al., 1987). Later studies by Bradbury, et al. using NMR demonstrated residues 31 to 95 of H2A and 37 to 114 of H2B to be crucial in forming the H2A-H2B dimer. Furthermore, residues 42 to 120 of H3 and 38 to 102 of H4 were detected to be involved in the tertiary structure of the $(H3\text{-}H4)_2$ tetramer (Wu, et al., 1987). Bonaduce, et al., found that single specific substitutions at residue 13 and 42 were lethal in yeast *Saccharomyces cerevisiae* (Bonaduce, et al., 1989). Studies employing trypsin-cleavage techniques have disclosed that the amino terminal regions of the core

histones are nonessential for histone-histone interactions while at least the carboxyl terminus of H2A is implicated in the formation of the dimer-tetramer association in the octamer (Wu, et al., 1987).

Current Model Systems

There have been numerous biochemical and physical studies performed on the nucleosome and the nucleosome core particle. Contrary to most proteins, no known histone mutations are known to occur in nature. By specific genetic altering of prescibed regions of individual or paired histones, greater insight of the precise role these entities play in the cell should ultimately emerge.

There are two genetic approaches currently used in studying histones. A reverse genetic approach focuses on engineering a specific genetic mutation within a given histone gene *in vitro* with subsequent investigation *in vivo*, noting any phenotypic alterations associated with such changes. The alternative forward genetic approach relies on subjecting whole cells to some mutagenic agent such as ultraviolet light, observing any phenotypic changes that arise from the procedure and then identifying the cause of the new phenotype as being due to a histone mutation. Current work utilize a reverse genetics approach since the phenotype for a histone mutation is difficult to predict. Such methods can provide information regarding which specific sequences or regions of the histone gene are

involved with or required for specific functions. Through such genetic investigations combined with studies from x-ray diffraction, a clearer understanding of histone function can hopefully emerge. To date, very little molecular research has been performed on histone H3 although focus is beginning to concentrate on this histone (M. M. Smith, Personal Communication). Conversely, numerous studies have been performed on histones H2A, H2B and H4 (Schuster, et al., 1986; Wu, et al., 1987; McGee, et al., 1990).

Frameshift mutations of the two histone H2B genes (H2B1 and H2B2) have been introduced into Saccharomyces cerevisiae by transformation and recombination (Rykowski, et al., 1981). The results by Rykowski, et al. showed that both single mutant strains (carrying either the H2B1 or H2B2 frameshift mutation) were found to be viable and could undergo all phases of the yeast life cycle, demonstrating that yeast cells lacking one of the H2B variants can survive. Therefore each of the H2B genes are interchangeable. However, when H2A-H2B mutants are both present, the yeast can germinate and bud once and are incapable of further growth. Later studies were initiated which utilized a background lacking in wild-type H2B proteins (Wallis, et al., 1983). These experiments revealed that although the amino terminus of the two H2B histones are highly conserved, deletions of large segments of the N-

terminus of H2B does not affect the viability of the cell in vivo (Wallis, et al., 1983). Furthermore, these studies demonstrated that large C-terminal deletions of histone H2B were lethal, suggesting a vital role for the carboxyl regions of this histone.

Like H2B, each of the H4 genes are interchangeable (B. Mittman, Thesis). Deletion analysis by oligonucleotide-directed mutagenesis of histone H4 has revealed that much of the amino terminus and a smaller portion of the carboxyl terminus is not essential for cell sustenance (Kayne,et al., 1988). Conversely, sequences in the hydrophobic core region of histone H4 have been shown to be necessary for cell viability (Kayne, et al., 1988). Interestingly, unlike the H2B deletions, the G2 phase is irregularly extended and chromatin structure varies with large N-terminal deletions of H4 (Kayne, et al., 1988).

Further studies using direct point mutations have permitted additional information regarding structure-function relationships of the amino terminus of histone H4, particularly regarding the post-translational modification, acetylation (Megee, et al., 1990). Four lysines at positions 5, 8, 12, and 16 in histone H4 are known to undergo reversible acetylation at their ε-amino groups which has been associated with the commencement of several nuclear functions such as transcription, replication, and chromatin assembly (Megee, et al., 1990). The essential

role of these four lysines residues was demonstrated by Megee, et al., whereby alteration of these residues were either lethal to the cell or resulted in significant phenotypic changes. Interestingly, this research revealed that no individual lysine residue was vital for cell viability but collectively, the mutational effects proved lethal, indicative perhaps of significant modification of histone-DNA and/or histone-histone associations needed for normal functions (Megee, et al., 1990).

Our Current Model System

Using a reverse genetic approach, the initial goal of this project was to determine whether the yeast Saccharomyces cerevisiae with all chromosomal copies of the core histone genes made nonfunctional, could be rescued by a plasmid carrying the wild type copies of the four core histone genes. This plasmid could then be made using various combinations of histone mutations in order to investigate precise histone-histone interactions as a long range goal.

The first step for this work would involve construction of the yeast vector pRH1 (figure 2) from plasmids pTS101-2 (figure 3) and pBAM10/HHF1 (figure 4). pRH1 (figure 2) would carry the genes encoding the histones H2A, H2B, H3 and H4 as well as the gene for tryptophan. The nutritional markers (tryptophan and uracil) would provide a tracking mechanism for each plasmid throughout this work.

This vector would be transformed into a haploid yeast strain BAM 200 (figure 5) which contains H3 and H4 deletion mutations and ura 3-52 and tryp 1-289 mutations. BAM 200 (figure 5) would survive on plasmid borne copies of H3-1 and H4-1 and would contain a uracil gene correcting the cellular defect. The resulting strain BAM 201 (figure 6) would then be grown on ura+/tryp- media to promote loss of pBAM8/476 (figure 7) so that the newly produced strain, BAM 202 (figure 8), would survive on pRH1 (figure 2) exclusively.

The next step would involve construction of a yeast strain carrying defects in all chromosomal copies of the histone genes. BAM 202 (figure 8) would be crossed with TSY-157 (figures 9 and 10) which contains frameshift mutations of H2A-1, H2B-1, H2A-2, and H2B-2 but survives on the vector pTS101-2 (figure 3). The resulting diploid cell (figure 11) would then be grown on ura+/tryp- media to promote selective loss of pTS101-2 (figure 3), subsequently resulting in a diploid cell that solely contains the pRH1 vector (figure 12). After sporulation and tetrad dissection (figure 13), a plasmid dependence test (figure 14) would be performed to identify that the cell carries nonfunctional chromosomal copies of the histones and is rescued by pRH1 (figure 2). The plasmid dependence test (figure 14) initiates nonselective growth of yeast cells no longer containing the pTS101-2 vector from growth in tryptophan

rich media. Consequently, the resulting cell populations could be attributable to either cells containing combinations of functional and nonfunctional histone gene copies yet all presumably containing pRH1 exclusively. Further selective growth conditions would confine the possible cell populations to containing all chromosomal copies of the histone genes nonfunctional yet surviving on plasmid borne copies of pRH1. Finally, a Southern blot analysis (figure 14) would be performed to confirm the identity of this yeast strain construct.