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A reverse genetics approach for studying histone H4 structure-function relationships in yeast

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ABSTRACT

A previously constructed mutation of the histone H4 gene has been the subject of a detailed analysis. The mutation involves an H4 gene encoding two amino acid changes which substitute aspartic acid for glycine at positions 13 and 42 of the polypeptide. These substitutions result in a nonfunctional H4 protein when assayed in Saccharomyces cerevisiae. In contrast, an analysis of an amino terminal deletion of amino acids 3-26 results in a functional H4 protein. Physical biochemistry studies identify the sites of histone-histone interactions between residues 38-102 of histone H4, the same area in which position 42 resides. The question being asked is: Is the amino acid substitution at position 13 and/or position 42 responsible for rendering histone H4 nonfunctional? And if H4 is functional with a single amino acid change, are there any phenotypes associated with the mutation?

Two new H4 genes were constructed in vitro, each encoding a single amino acid substitution. The first gene (hhf1-9) substitutes aspartic acid for glycine at position 13 of the histone H4 and the second gene (hhf1-10) encodes an identical substitution at position 42. When assayed in yeast deleted for both chromosomal gene sets, both genes encoded for nonfunctional proteins resulting in a loss of histone function. This analysis indicates that the amino acids at positions 13 and 42 play critical structural roles in rendering the histone H4 protein functional.

A REVERSE GENETICS APPROACH

FOR STUDYING HISTONE H4 STRUCTURE-FUNCTION

RELATIONSHIPS IN YEAST

by

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INTRODUCTION

The nucleosome is the primary repeating unit of DNA organization. Evidence for the existence of nucleosomes, and accounts of earlier investigations of their properties, are summarized in a number of reviews (Elgin & Weintraub, 1975; Kornberg, 1977; Chambon, 1978; Felsenfeld, 1978; Lilley & Pardon, 1979).

The most prominent proteins associated with metaphase chromosomes and with interphase DNA are the histones. Insight into the structural role of histones was obtained with the discovery of the repetitive structure of chromatin by Hewish and Burgoyne in 1973. They and, subsequently, others reported that digestion of chromatin by certain enzymes, such as micrococcal nuclease, yields DNA fragments that are approximately 200 base pairs in length or multiples thereof (Hewish & Burgoyne, 1973; Noll, 1974; Shaw et al., 1976). They also suggested that chromatin consists of repeating units, each of which is protected from enzymatic cleavage except where any two units join each other. Such information suggested regular spacing arrangements in chromatin.

About the same time as the discovery of the nucleosome, electron microscopy provided visual evidence concerning the repetitive structure of chromatin (Woodcock, 1973; Olins & Olins, 1974). Ada and Donald Olins reported electron microscopic observations of chromatin fibers composed of linear arrays of spherical particles. The particles occur regularly along the axis of a chromatin strand and are approximately 70A in diameter. These particles, which resemble beads on a string, are now referred to as nu (ν) bodies or nucleosomes. These findings conform to

the earlier proposal which suggested the existence of repeating units and are supported by both the analysis of enzymatic digestion of chromatin (Hewish & Burgoyne, 1973) and X-ray diffraction studies (Lilley & Pardon, 1979).

In 1975, Roger Kornberg published the results of his study of the precise interactions of histone molecules and DNA in chromatin (Kornberg, 1975). His work showed an octameric protein complex made up of a central H3-H4 tetramer and two H2A-H2B dimers. These data correlate well with the two previous observations and provide the basis for a model which later explains the 1:1 mass ratio of histones to DNA in chromatin.

Throughout the past decade the nucleosome structure has once again been thoroughly reviewed (Isenberg, 1979; Lilley & Pardon, 1979; McGhee & Felsenfeld, 1980; Igo-Kemenes et al., 1982; Pederson et al., 1986; Wu, et al., 1986). The structure of the core particle has been intensively studied by scattering, neutron diffraction, electron microscopy, protein-protein and protein-DNA cross-linking, and spectroscopic and enzymatic probes (Belyavsky et al., 1980; Bentley et al., 1984; Brandt et al., 1980; Finch et al., 1977; Klug et al., 1980; Martinson & True, 1979; Mirzabekov et al., 1978; Moss et al., 1976; Urban et al., 1979). Such a thorough approach to uncovering structural and perhaps functional properties of histone-histone and histone-DNA interactions is sure to be the forerunner of many related studies on specificity in form and function at the level of the nucleosome.

Nucleosome Organization

The most detailed information on nucleosome organization and its interactions with DNA is presently being determined by X-ray crystallo-

graphy (Burlingame et al., 1985; Klug et al., 1985; Richmond et al., 1984). Several models have been proposed for the structure of the nucleosome core particle. All models show an octamer of histones made up of a central H3-H4 tetramer and two H2A-H2B dimers. Approximately 146 base pairs of DNA are wrapped around the core particle in two right-handed B-DNA superhelical turns.

Richmond et al., 1984, have introduced a 7A resolution structure of the nucleosome core particle. The shape of the core particle is described as wedge-like with a bipartite organization. It is reported to be 57A thick and 110A in diameter.

In contrast to the above, Burlingame et al., 1984, have determined a 3.3A resolution structure for the core histone octamer. They found the shape of the octamer to be roughly that of a rugby ball with a tripartite organization. The overall shape of the octamer is a prolate ellipsoid 110A in length and 65-70A in diameter; radically different from those determined in earlier studies.

Histone-Histone Interactions

A hierarchy of pairwise interactions among histones was defined by Isenberg and co-workers (Isenberg, 1979). Their <u>in vitro</u> physiochemical analysis of the interaction between calf thymus histones reported that strong interactions occur between H2A and H2B and between H2B and H4 to form dimers; and between H3 and H4 to form a tetramer. There are weak interactions between H2A and H4 and H2B and H3, with an intermediate interaction, not much weaker than the strong ones, between H2A and H3. Their analysis was repeated and confirmed for the histones of the pea plant, yeast, and Tetrahymena, as well as for heterospecific mixtures of

histones (Spiker & Isenberg, 1977; Mardian & Isenberg, 1978; Glover & Gorovsky, 1978).

Cross-linking of chromatin by tetranitromethane results in the formation of H2B-H4 (Martinson & McCarthy, 1975), UV irradiation leads to H2A-H2B linking (Martinson et al., 1976), and H3 is linked to H4 by carbodiimide (Bonner & Pollard, 1975). All of these cross-linkers are of zero length, and such cross-linking implies that the histones that are linked had previously been in contact, and hence presumably interacting.

Histone Complexes

Bradbury and co-workers have conducted a series of elegant experiments designed to determine which part of each histone is involved in complex formation (Bradbury et al., 1977; Moss et al., 1976; Bohm et al., 1977). Utilizing NMR to study the ability of specific fragments of the core histone to interact, they concluded that histones H2A and H2B interact to form a dimer in the regions corresponding to residues 31-95 for H2A and 37-114 for H2B. Similarly, H3 and H4 interact to form a tetramer through the region extending from residues 42-120 for H3 and 38-102 for H4. Thus the N-terminal regions, and in some cases a short region near the C terminus, are not required for complex formation. These data are consistent with a model for the histone core in which the larger part of each histone and the major sites of histone-histone interactions reside in globular regions, with the more mobile N-terminal regions extending as relatively unstructured "tails" (McGhee & Felsenfeld, 1980). When the N-terminal tails are cleaved by digestion with trypsin the particle partially unfolds (Lilley & Tatchell, 1977), showing the importance of surface charge on the globular component of the histones as well as

their N-terminal regions in maintaining structural integrity.

NMR studies have also shown that central regions of the histone polypeptides are involved in histone-DNA interactions, while the high basic amino termini of H2A and H2B are free and mobile, and the amino termini of H3 and H4 are only weakly bound (Cary et al., 1978). Although the data indicate that most of the histone is located in the hydrophobic protein core, approximately 25% of the protein is located external to this core, a value quite similar to the percent of trypsin-sensitive residues in the histones.

Histone Modifications

The four core histones undergo five major types of postsynthetic modification: acetylation, methylation, phosphorylation, ADP-ribosylation and, in the case of histone H2A, covalent linkage to the protein ubiquitin (Isenberg, 1979; McGhee & Felsenfeld, 1980; Wu et al., 1986). No attempt is made here to repeat the material covered in those reviews, and subsequent information is mentioned only as it appears important to the understanding of H4.

Histone H4

Histone H4 is an arginine-rich species and is highly conserved throughout evolution. The amino terminal region is extremely basic, whereas the carboxyl region has an amino acid distribution characteristic of globular proteins and is involved in histone-histone interactions. At present no structural role has been demonstrated for the amino-terminal portion.

Histone Genes

In contrast to the multiple copies of the histone genes in most

eukaryotic organism, yeast cells contain only two copies of each histone gene. Smith and colleagues have cloned and characterized the complete DNA sequences of the two H3-H4 gene loci (Smith & Andresson, 1983). They reported four nucleotide substitutions between the H4 coding sequences of the copy-I and copy-II genes. All four substitutions occured in the third base position which result in synonomous codons and therefore both code for identical H4 polypeptides.

Since haploid yeast can survive in the presence of a single histone H4 gene, yeast strains have been constructed that are deleted for copy-I or the copy-II set of the H4 genes. The two gene sets are unlinked therefore segregate independently during meiosis. After transformation, yeast deleted for both chromosomal gene sets and surviving solely on a plasmid borne copy of the H4 gene may be obtained. This double deletion results in a loss of histone function and can be verified by a plasmid dependence test. The test relies on the fact that minichromosomes which contain a cloned centromere are less stable than the normal yeast chromosomes containing a centromere (Fitzegerald-Hayes, et al., 1982; Stinchcomb, et al., 1982; Murray & Szostak, 1983; Clarke & Carbon, 1985). The test can then be united with a plasmid shuttle system that allows us to analyze the effects of an H4 mutation.

As a model eukaryotic organism, yeast has proven to be useful for investigating basic problems in genetics and molecular biology as well as occupying an important position in modern biotechnology. Yeast will be used in this project because of its advantages for molecular and cellular analysis. Such advantages are (1) the simplicity of histone gene organization in yeast, (2) yeast can be transformed with foreign DNA, (3)

genetic analysis can be performed on colonies of individual isolates and (4) a shuttle vector system is available for analysis.

Histone Analysis

A system has been developed (Mittman, 1986) to analyze histone H4 mutations in yeast using "reverse genetics" (mutations constructed *in vitro* then analyzed *in vivo*). Site-directed sodium bisulfite mutagenesis of one of the H4 gene copies, *HHF1*, has been used to generate a variety of mutations in the H4 DNA sequence. One particular mutation involves an H4 gene encoding two amino acid substitutions. The substitutions of aspartic acid for glycine at positions 13 and 42 of the histone protein results in a loss of histone function when assayed in yeast (Mittman, 1986). In contrast, both an analysis of an amino terminal deletion for amino acids 3-26 and a single substitution of glutamic acid for glutamine at position 27 resulted in a functional H4 protein (Figure 1). Therefore, at least some change in the primary structure of H4 is tolerated. Also, it should be reiterated that position 42 resides in the same area in which H3 and H4 interact to form a tetramer.

The principle objective of this thesis is to determine whether both amino acid substitutions encoded by a histone H4 gene containing two mutations are responsible for loss of histone function in yeast, *Saccharomyces cerevisiae*. The question being asked is: Is the amino acid substitution at position 13 and/or 42 responsible for rendering the histone H4 protein nonfunctional? And if H4 is functional with a single amino acid change, are there any phenotypes associated with the mutation?

Since structure-function questions can be answered using techniques of *in vitro* mutagenesis coupled with *in vivo* analysis in yeast, the

described system provides a means for an indepth biologically significant analysis of chromatin architecture at a fundamental level. This architecture has an implicated role in cell growth, gene expression, and differentiation. Because nuclear organization of yeast is similar at a gross level to that in higher organisms, answers to questions asked in this relatively simple eukaryote can be extrapolated to more complex organisms like plants and animals.

MATERIALS

Strains and Growth Conditions

Bacterial strains were maintained 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) with 100ug/ml of ampicillin added for plasmid selection. Liquid cultures were grown in L-broth (1% Bacto Tryptone [Difco], 0.5% Bacto Yeast Extract [Difco], 0.5% NaCl, pH 7.2) supplemented with 100ug/ml ampicillin [Sigma] for plasmid growth. All bacterial strains were grown at 37C.

The strains of S.cerevisiae were grown at 30C in YPD (1% Bacto Yeast Extract, 2% Bacto Peptone [Difco], 2% dextrose) or defined synthetic medium (0.17% Yeast Nitrogen Base without Amino Acids [Difco], 0.5% ammonium sulfate, 2% dextrose) supplemented with required amino acids as described by Sherman et al., 1982. Strains were maintained on complex media agar plates with appropriate antibiotics needed for the maintenance of plasmids.

Enzymes and Nucleotides

Restriction enzymes and T4 ligase were purchased from Bethesda Research Laboratories, International Biotechnologies, Inc. or Promega Biotec and reactions were performed according to manufacturer's directions. Calf intestinal alkaline phosphatase and RNAase A were purchased from Boerhinger Mannheim (Mannheim, F.R.G.) and Sigma Chemical Company respectively.

Plasmids

Plasmids pBam8/HHF1 and pBam8/hhf1-2 were provided by B.A. Mittman. The plasmid pBam11/hhf1-5 was a gift from E.R. Harkrader. (University of Richmond).

METHODS

Plasmid Preps

One bacterial colony containing the plasmid of interest was grown overnight in 20ml of L-amp. A 1:100 dilution of the overnight culture was used to inoculate 400ml of fresh L-amp which was grown for 4 hours at 37C with shaking. Two mls of a 34mg/ml solution of chloramphenicol [Sigma] in 95% ethanol were added and cells were grown for an additional 16 hours at 37C. The cells were centrifuged in a Sorvall GSA rotor at 6000rpm for 10 minutes and resuspended in 4ml of 25% sucrose solution with 50mM Tris pH 7.5. Two mls of lysozyme (10mg/ml in water) were added to the cells and incubated on ice for 5 minutes. Two mls of 0.5M EDTA (pH8.0) were added followed by an additional 5 minutes incubation on ice. Lysis solution [5ml of 0.1% Triton X-100, 62mM EDTA, 50mM Tris (pH 8.0)] was added and the cells were left on ice for 20min to ensure thorough lysis. The mixture was then centrifuged for 40min at 12,000rpm in a Sorvall SS34 rotor to pellet cellular debris. The supernatant was mixed with 1g/ml of CsCl and a 1/10 vol of EtBr (5mg/ml) and then ultracentrifuged at 40,000rpm at 18C for 40hrs in a Beckman Ti50 rotor.

The plasmid DNA (lower band) was recovered from the centrifuge tube with a syringe, dialyzed for one hour against 10mM Tris (pH 7.5), 0.1mM EDTA (1xTE) and phenol extracted. The DNA was then dialyzed twice more with fresh 1xTE at 8hr intervals (Maniatis et al., 1982).

Mutant H4 Gene Construction

In order to construct the genes encoding a mutation at residues 13 or 42 of the H4 protein, plasmid DNA was isolated from cultures of bacteria containing the plasmid of interest (pBam8/HHF1 [wild type] and pBam8/hhf1-2 [mutant with the 2 nucleotide substitutions]). The plasmid preparations were digested with the restriction enzyme BamHI to yield 488 base pair fragments which contained the H4 gene. A DEAE-cellulose NA45 membrane [Schleicher & Schuell] was used to extract the fragments from a 1% agarose gel. The membrane was exposed to 0.5ml of a high salt buffer (10mM Tris pH 7.4, 1mM EDTA, 1.5M NaCl) and incubated 15 minutes at 65C with intermittent vortexing to aid in DNA release. After the membranes were removed 1ul of E. coli t-RNA [Sigma] along with 95% ETOH was added to the DNA. Samples were placed at -70C for 15 minutes. The pellets were washed twice with 70% ETOH, dried and redissolved in the appropriate volume of TE.

The HHF1 and hhf1-2 genes were cut with a second restriction enzyme, BanI, which isolated each half of the H4 gene. Gene fragments were resolved by electrophoresis on a polyacrylimide gel (Maniatis et al., 1982). The gel was placed in a staining buffer (10:1 H20:1xTE buffer with Ethium Bromide [0.5ug/ml]) for approximately 30 minutes. The DNA bands were excised from the gel, recovered in elution buffer (500mM Ammonium Acetate, 10mM Magnesium Acetate, 1mM EDTA) and ethanol precipitated (Maxam & Gilbert, 1980). Samples were placed at -70C for 1 hour, washed with 75% ETOH, centrifuged at 10,000rpm for 5 minutes, and dried.

The gene fragments encoding amino acid substitutions at positions 13 or 42 were ligated with (T4 ligase) and a wild type gene fragment from

the appropriate counterpart gene (Figure 2). These newly constructed H4 genes were ligated into new plasmids that served as cloning vehicles for yeast transformation.

Vector Constructions

Plasmid pBam11 was digested with BamHI for 2hrs at 37C. One ul of calf intestinal alkaline phosphatase (Chaconas and van de Sande, 1980) was added and incubated an additional 30 minutes at 37C. After the enzyme and phosphatase digestions were complete, 150ul of TE along with 200ul of neutralized phenol were added, vortexed briefly and centrifuged 2 minutes to separate the aqueous and phenol phases. The top aqueous layer, which contained the DNA, was removed. A 1/10 vol (approx. 18ul) of 3M sodium acetate pH 5.5 and 2.5 volumes (approx. 0.45mls) of 95% ETOH was added to the phenol extracted DNA sample. In order to precipitate the DNA, the sample was stored at -70C for 1 hour then centrifuged for 15 minutes. The DNA pellet was washed in 70% ETOH and dried.

Plasmids pBam11/hhf1-9 and pBam11/hhf1-10 were constructed by ligating the 488-bp mutant H4 genes into the BamHI site of plasmid pBam11 (Figure 3). This vector is a derivative of pBam11/hhf1-5 (Harkrader, 1988) and differs only in the absence of the H4 gene.

Plasmid Mini Preps

A 1.5ml sample of transformed E. coli cells were spun in a microcentrifuge for 1 minute. The supernatant was removed and the pellet resuspended in 100ul of an ice-cold solution of 50mM glucose, 10mM EDTA, and 25mM Tris Cl (pH 8.0). After storing for 5 minutes at room temperature, 200ul of a freshly prepared solution of 0.2N NaOH, 1% SDS was added to lyse the cells. The sample was mixed by slow agitation and

stored on ice for 5 minutes. The proteins were precipitated out of the cell mix with 150ul of 3M sodium acetate (pH 5.5). After centrifuging for 5 minutes the supernatant (approx 400ul) was transfered to a fresh tube. An equal volume of phenol/chloroform was added, vortexed, and mixed by microcentrifuging for 2 minutes. The aqueous upper phase, which contains the DNA, was transfered to a fresh tube. Two volumes of 95% ETOH were added, vortexed, left standing at room temperature for 2 minutes, and finally microcentrifuged for 5 minutes. Samples were washed with 70% ETOH and dried.

H4 Orientation

Proper orientation of the gene fragments (hhf1-9 and hhf1-10) into the BamHI site of pBam11 was verified by gel electrophoresis. The newly constructed plasmids, along with a wild type plasmid and a plasmid containing no H4 gene, were digested with the restriction enzyme BanI.

The BanI site located within the H4 gene fragment was the key to verification. If the H4 gene was inserted in the proper orientation the H4-BanI site would be located 553bp downstream from the previous BanI site located at position 3480. If the H4 gene was inserted improperly the H4-BanI site would be 840bp down stream from the same previous BanI site located at position 3480 (Figure 4). When resolved on a polyacrylimide gel both genes (hhf1-9 and hhf1-10) yielded 553bp DNA fragments. This was in agreement with the wild type plasmid and confirmed proper orientation. The plasmid with no H4 gene yielded one less DNA fragment due to the absence of the BanI site located in the departed H4 gene.

Bacterial Transformation

An overnight saturated culture of JM83 was diluted 1:25 in L-broth

and grown at 37C overnight. Cells were pelleted by centrifugation at 6000rpm for 10 minutes. The cell pellet was resuspended in a 1/2 volume of 0.1M MgCl2 and repelleted by centrifugation. The final pellet was resuspended in a 1/15 volume of 0.1M CaCl2 and left on ice for 30 minutes to allow the cells to become competent.

The DNA ligation mix (10-500ng of plasmid+insert) was added to 0.2ml of competent cells and left on ice an additional 30 minutes. Transformation mixes were heat shocked at 42C for 2 minutes and 0.8ml of L-broth was added. After incubating at 37C for 30 minutes, 0.5ml of the cells were spread on L-ampicillin plates and incubated at 37C.

Yeast Transformation

An overnight saturated culture of yeast cells were diluted 1:1000 in YPD broth and grown at 30C overnight. Cells were pelleted and washed twice in a 1/5 volume of 10mM Tris-HCl (pH 7.5), 0.1mM EDTA. A 1/10 volume of 0.1M lithium acetate in 10mM Tris-HCl (pH 7.5), 0.1mM EDTA was added and cells were incubated for 60 minutes at 30C. The cells were repelleted and resuspended in 2.5ml of 15% glycerol, 0.1M lithium acetate, 10mM Tris-HCl (pH 7.5), 0.1mM EDTA.

Approximately 5ug of plasmid DNA in a 10ul volume was added to 0.3ml of competent cells. A 0.7ml addition of 50% PEG 4000 was made and tubes incubated at 30C without shaking for 60 minutes. Cells were incubated at 42C for 5 minutes, cooled to room temperature, and pelleted. The transformation mixes were washed twice in minimal media, spread on minimal media agar plates and incubated at 37C.

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 was not just due to sampling error.

RESULTS

Two new H4 genes were constructed *in vitro*, each encoding a single amino acid substitution. This was done by isolating the plasmids pBam8/*HHF1* (wild type) and pBam8/*hhf1*-2 (mutant with the 2 nucleotide substitutions) from cultures of bacteria. Plasmid preparations were digested with the restriction enzyme *Bam*HI to yield 488 base pair fragments which contained the H4 gene. The *HHF1* and *hhf1*-2 genes were cut with a second restriction enzyme, *Ban*I, which isolated each half of the H4 gene.

The next step involved ligation of gene fragments encoding for amino acid substitutions at positions 13 and 42 with a wild type gene fragment from the appropriate counterpart gene resulting in 2 new mutant genes. The first gene (*hhf1*-9) substitutes aspartic acid for glycine at position 13 of the histone protein. The second gene (*hhf1*-10) encodes for an identical sub- stitution at position 42. When assayed in yeast deleted for both chromosomal gene sets, both genes encoded for nonfunctional proteins resulting in a loss of histone function. This analysis indicates that amino acids at positions 13 and 42 play a role in rendering the histone H4 protein functional.

Genetic Assay for H4 Genes

Each mutant gene contains a recognition sequence at both ends for the restriction endonuclease *Bam*HI. The genes were ligated into the *Bam*HI site of plasmid pBam11 to construct the new plasmids pBam11/*hhf1*-9 and pBam11/*hhf1*-10. The newly constructed plasmids contain the selectable marker uracil, which allows for the identification of transformants. In

addition, the plasmids carry an autonomously replicating sequence (ars4)(Bouton & Smith, 1986) and the centromere (CEN4) from chromosome IV of yeast (Clarke & Carbon, 1985). The CEN4 and ars4 sequences give the plasmid several unique features. (1)An origin of DNA replication is provided by ARS. (2)The copy number of the plasmid is maintained at an average of approximately one per cell. (3)The plasmid is relatively stable during mitosis and segregates like a single genetic locus during meiosis. Finally, (4)the centromere also prevents the stable integration of the plasmid into the yeast genome (Clarke and Carbon, 1985).

Yeast Strain Mutations

The mutated yeast strain used (strain 200) is unable to produce the nucleotide uracil and the amino acid tryptophan, both essential for cell survival. In order to compensate for this deficiency the cells must have the biosynthetic block corrected by a plasmid carrying a gene which corrects the chromosomal nutritional defects or be grown in media containing these nutrients.

The pBam11 plasmids which carry the mutant H4 genes along with the selectable marker uracil were used to transform a haploid yeast strain deleted for both chromosomal gene sets of H3-H4 and are dependent on the plasmid pBam10 containing a wild type H3-H4 gene and the selectable marker tryptophan. The yeast cells carrying both plasmids are now able to synthesize their own uracil and tryptophan and take part in the plasmid shuttle system (Figure 5).

Plasmid Shuttle System

The system is designed to "shuttle in" the mutated plasmid, and to "shuttle out" the wild type plasmid, provided the cell can survive with

only the mutated allele of the H4 gene (Harkrader, 1988).

Histone H4 Mutations Analyzed

The plasmid dependence test allows us to analyze the effects of an H4 mutation in a genetic background that completely lacks a wild-type H4 protein (Figure 5). This was done by plating the transformed yeast cells on minimal SD media without uracil and tryptophan (SD -ura -trp). URA+ TRP+ colonies were serially transferred five times in SD -ura broth in an attempt to lose plasmid pBam10 (contains wild type H4 and trp gene). After plating on SD -ura plates, cells were replica plated on SD -ura -trp plates. In the absence of chromosomal copies of the H3 and H4 genes every colony that grew on the SD -ura plates also grew on the SD -ura -trp plates since every viable cell contained the wild type plasmid along with the trp gene. Cells which never grew on the SD -ura plate from the onset presumably lost the wild type plasmid and were unable to survive with the mutant H4 gene.

If a cell lost its wild type plasmid and was able to survive with the plasmid containing the mutant gene, the cell would be viable on the SD -ura plate but inviable when plated on SD -ura -trp. This condition never occured. The results (Table I) indicate that all cells viable on the SD -ura plates, were also viable on the SD -ura -trp plates. The hypothesis test for two population proportions at a 5% significance level (Weiss and Hasset, 1987), along with the proportion of cells which retained the wild type plasmid (100%), leads to the conclusion that both mutant genes encode for nonfunctional proteins resulting in a loss of histone function.

Reverse Shuttle Experiment

Due to the relatively new development of the shuttle system, a "reverse shuttle" experiment was performed. This experiment was designed to confirm our results and test the system's effectiveness for shuttling histone H4 alleles in and out of the yeast cell.

Yeast cells from the above experiment carrying both plasmids were grown in SD -trp broth in an attempt to lose the plasmid pBam11/hhf1-9 (contains mutant H4 and ura gene). After plating on SD -trp plates, cells were replica plated on SD -trp -ura plates. Less than 50% of the colonies that grew on the SD -trp plate grew on the SD -trp -ura plate following the replica plating. The results (Table I) indicate plasmid shuttling does occur and yeast colonies are recovered when functional H4 protein is still present. These results agree with those reported by E.R.L. Harkrader (Harkrader, 1988).

DISCUSSION

A system has been developed that allows us to construct a mutation in histone H4 and analyze the effect in vivo (Mittman, 1986). To do this demands the absence of any wild type complementing H4. Due to the highly reiterated nature of the histone genes (Kedes, 1979), it is not possible to provide such a background in most organisms. However, yeast cells contain only two copies of the histone H4 gene and can survive in the presence of only one functional copy of the gene. Mutations (called hhf1-9 and hhf1-10) were transformed into yeast cells to determine if the effects of a single amino acid substitution encoded by a histone H4 gene was responsible for a nonfunctional histone. 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.

At the onset of this research, predictions were made regarding the ability of the histone H4 protein to tolerate a change at residues 13 and 42 of the polypeptide chain. The amino acid change substituted aspartic acid for glycine at both positions. We were predicting that the change at residue 13 may result in a functional H4 protein since this region can be deleted from an H4 protein and still produce a functional polypeptide (Mittman, 1986). Residues 42 of H4 is located within the identified region of histone complex formation (area in which H3-H4 interact to form a tetramer), therefore we felt this change may disrupt proper histone-histone interactions resulting in a nonfunctional protein. Both substitutions resulted in nonfunctional proteins.

Core Particle Deletions

It has long been suspected that the highly basic amino terminal regions of the histones are the primary sites of interaction with DNA and that their central, more globular regions are sites for interactions with other histones. Grunstein and co-workers have recently used yeast genetics to address this question and found an unexpected result. There are two genes for both H2A and H2B in yeast. Either of these subtypes can provide proteins for a normal or nearly normal phenotype (Rybowski et al., 1981; Kolodrubetz et al., 1982): thus the amino acid differences between the subtypes for each histone seem to have no detectable effect on protein function. Furthermore, using in vitro mutagensis of H2B genes on plasmids in yeast lacking both H2B subtypes, they found that deletion of up to 20 amino acid residues (positions 3-22) of the amino terminus of H2B has no apparent effect on the synthesis or function of this histone in vivo, or on the phenotype of the cells (Wallis et al., 1983). However, mutations which caused not only deletion within the unstructured amino-terminal region, but also in the hydrophobic structured core of this protein (residues 17 to 70), were found to be lethal (Wallis et al., 1983). Histones lacking amino terminal regions (removed by tryptic proteolysis) had previously been shown to be capable of refolding core particle-length DNA (Whitlock & Stein, 1977), but the finding that a protein lacking a highly conserved region of distinctive character could function throughout the cell cycle and through meiosis was unexpected.

In the case of H4, both an analysis of an amino terminal deletion for amino acids 3-26 and a single substitution of glutamic acid for glutamine at position 27 resulted in a functional H4 protein. While these

results may be rationalized in that the amino terminus of H4 resides on the outside of the octamer and has little role in the formation and maintenance of the nucleosome structure, we should not conclude that the amino terminus has no role in chromatin organization.

Complementation of Amino Termini

The in vitro studies with trypsin-treated chromatin suggest the histone tails are not essential for the formation or maintenance of nucleosome structure. However, trypsin treatment has been shown to destroy the higher-ordered solenoid structure of native chromatin (Allan et al., 1982). It is not clear if the basic tails of all four histones act together in forming the solenoid or if different groups have different functions. Thus it is possible that the amino terminus of H4 has a role in higher orders of folding that can be complemented by the other core histones. If this were the case one could predict that the removal of the basic tail of a different histone may by itself be tolerated but the cumulative effect of more than one histone deletion could destroy chromatin structure. These experiments are currently underway with H2B and H2A by Grunstein and co-workers. They have shown that switching portions of the N termini between H2B and H2A has created two chimeric histone proteins within the same cell and has no obvious effect on viability (Schuster et al., 1986). This supports the argument that the charged N-termini of the two histones are to some extent interchangeable and complement each others function.

The H3-H4 complementation story will be different since an organism surviving with an amino terminal deletion of H4 is not as vigorous as the wild type. Experiments designed to examine H2A-H2B & H4 complementation

are in progress (Heljams and Swardlow).

Evolutionary Changes

An attempt was made to gather, align and briefly analyze all histone sequences available in the NIH-GENbank, including all recently published sequence data not currently available in this data base (Wells, 1986). Data from direct amino acid sequence analysis along with data from translated gene sequences were presented for the histone subtype H4 leading to a consensus sequence (Figure 6).

For yeast, no evolutionary changes have occurred at positions 13 or 42. In fact, from the H4 sequence list compiled, only one sequence (Tetrahymena H4) showed an amino acid replacement (valine for glycine at position 13) at either position 13 or 42. The known result that the entire amino terminus of histone H4 of yeast differs from the H4 consensus sequence by only one amino acid replacement (position 21) establishes H4 as a highly conserved molecule. The conservation of the entire sequence suggests that all of it must be important in the function of the protein, and lends support to the concept of a common basis for chromatin structure.

Rationale & Suggested Avenue

More than 50 years ago, T.H. Morgan recognized that "favorable material" for investigating the mechanisms of cell differentiation would be a simple organism in which the development could be analyzed at the level of single genes and single cells (Morgan, 1934). Although no eukaryotic organism as yet is as well studied and well understood as E. coli, the combination of genetic and molecular techniques now available for experimentation in S. cerevisiae is unmatched by any other eukaryotic

organism. The development of a trans-formation system (Beach and Nurse, 1981) has made possible the cloning of virtually any gene for which a mutant has been identified by complementation. Advances in recombinant DNA technologies applicable to yeast, such as gene substitution, permit sophisticated molecular technology to be coupled with classical genetic analysis.

By screening large numbers of specific H4 mutations it will be possible to determine specific residues which are critical to the function of the H4 protein. One approach is to generate a broad range of amino acid changes throughout the H4 protein to identify potential sites of interest. The second approach focuses on specific questions about particular modification sites. This harvested information will lead to a better understanding of the three dimensional structure of histone H4, its interactions with other histones, and its related functions within the cell.

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Table 1. Plasmid Shuttle Results.

TABLE I

Experiment	1	2	3	4
Plasmids involved	pBam11/*hhf1*-9 pBam10	pBam11/*hhf1*-10 pBam10	pBam11/*hhf1*-9 pBam10	pBam11/*hhf1*-10 pBam10
Plasmid attempting to lose	pBam10	pBam10	pBam11/*hhf1*-9	pBam11/*hhf1*-10
Frequency of cells losing plasmid	0/555	0/756	387/617	277/481
Percent of cells losing plasmid	0	0	62.72	57.58
Assumption	Both mutant genes *hhf1*-9 and *hhf1*-10 encode nonfunctional proteins resulting in a loss of histone function.		Plasmid shuttling does occur and yeast colonies are recovered when functional H4 is still present.	

Figure 1. Comparison of H4 Protein Sequences.

Amino acid sequence of wild type yeast histone H4 (HHF1) is compared to a sequence with a single amino acid substitution at position 27 (Mittman, 1986), an amino terminal deletion for amino acids 3-26 (Mittman, 1986), and two amino acid substitutions at positions 13 and 42 of the histone protein (Mittman, 1986). A substitution is represented by an asterisk. A dashed line in a sequence represents a deletion. The numbering refers to the sequence of the yeast proteins. The one-letter code for amino acids is: A-Ala, C-Cys, D-Asp, E-Glu, F-Phe, G-Gly, H-His, I-Ile, K-Lys, L-Leu, M-Met, N-Asn, P-Pro, Q-Gln, R-Arg, S-Ser, T-Thr, V-Val, Y-Tyr.

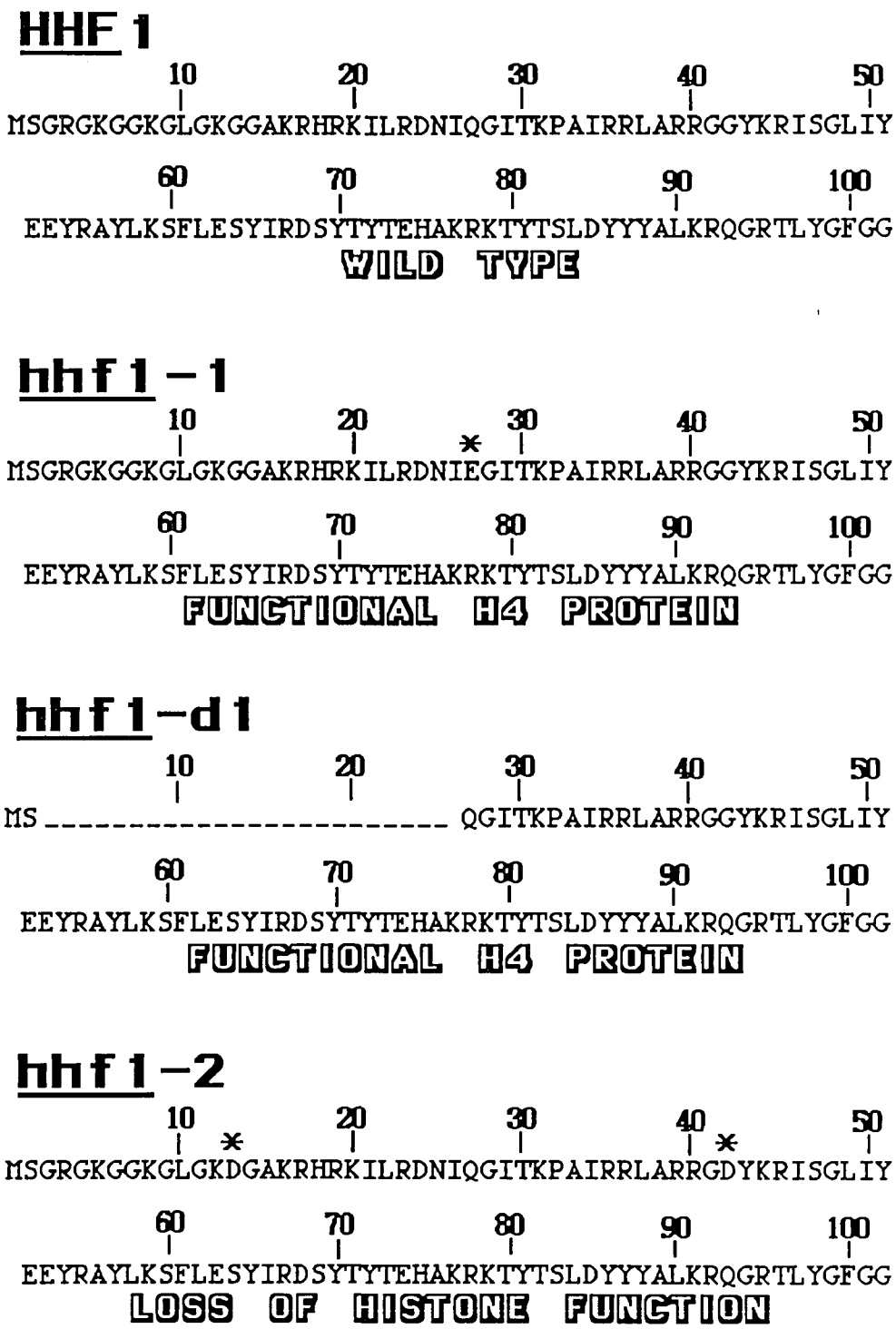

HHF 1
10 20 30 40 50
MSGRGKGGKGLGKGGAKRHRKILRDNIQGITKPAIRRLARRGGYKRISGLIY
60 70 80 90 100
EEYRAYLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGFGG
WILD TYPE
hhf 1 - 1
10 20 * 30 40 50
MSGRGKGGKGLGKGGAKRHRKILRDNIEGITKPAIRRLARRGGYKRISGLIY
60 70 80 90 100
EEYRAYLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGFGG
FUNCTIONAL H4 PROTEIN
hhf 1-d 1
10 20 30 40 50
MS---------- QGITKPAIRRLARRGGYKRISGLIY
60 70 80 90 100
EEYRAYLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGFGG
FUNCTIONAL H4 PROTEIN
hhf 1-2
10 * 20 30 40 * 50
MSGRGKGGKGLGKDGAKRHRKILRDNIQGITKPAIRRLARRGDYKRISGLIY
60 70 80 90 100
EEYRAYLKSFLESYIRDSYTYTEHAKRKTYTSLDYYYALKRQGRTLYGFGG
LOSS OF HISTONE FUNCTION

Figure 2. Mutant H4 Gene Construction

(a) The HHF-1 gene from the plasmid pBam8/HHF1; (b) the hhf1-2 gene from the plasmid pBam8/hhf1-2. The 488bp fragments were digested with BanI to isolate each half of the H4 gene. The gene fragments encoding amino acid substitutions at positions 13 or 42 were ligated with a wild type gene fragment from the appropriate counterpart gene resulting in 2 new mutant genes (c). The hhf1-9 gene substitutes aspartic acid for glycine at position 13 of the histone protein. The hhf1-10 gene encodes for an identical substitution at position 42.

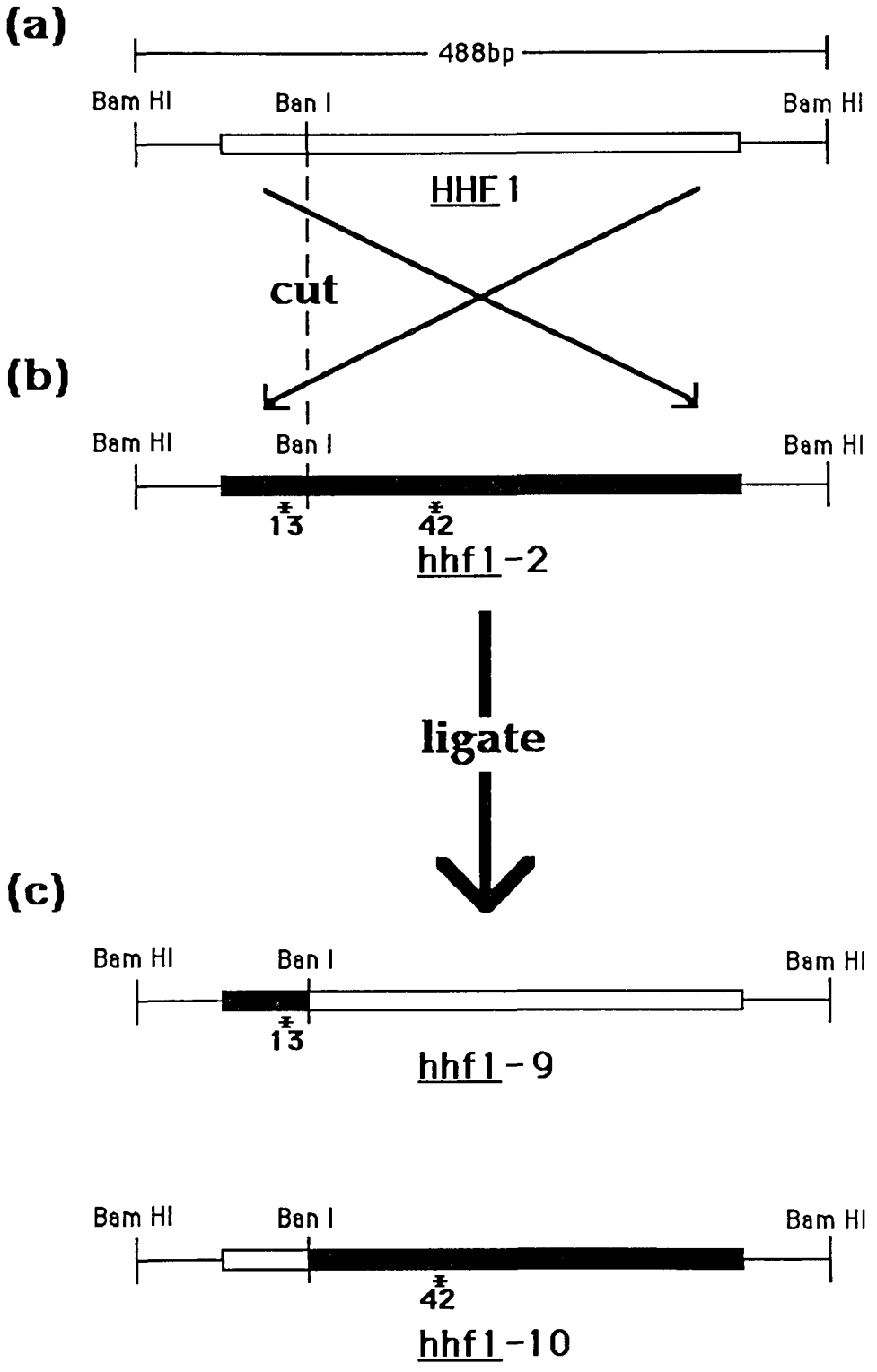

(a)
488bp
Bam HI
Ban I
Bam HI
HHF 1
cut
(b)
Bam HI
Ban I
Bam HI
13
42
hhf1-2
ligate
(c)
Bam HI
Ban I
Bam HI
13
hhf1-9
Bam HI
Ban I
Bam HI
42
hhf1-10

Figure 3. Vector Constructions

(a) Plasmid pBam11 was cut with the restriction enzyme BamHI. (b) The 488bp mutant H4 genes were ligated into the BamHI site of the vector yielding the plasmids pBam/hhf1-9 and pBam11/hhf1-10 (c). The one letter code for restriction enzymes is: B-BamHI, E-EcoRI, H-HindIII.

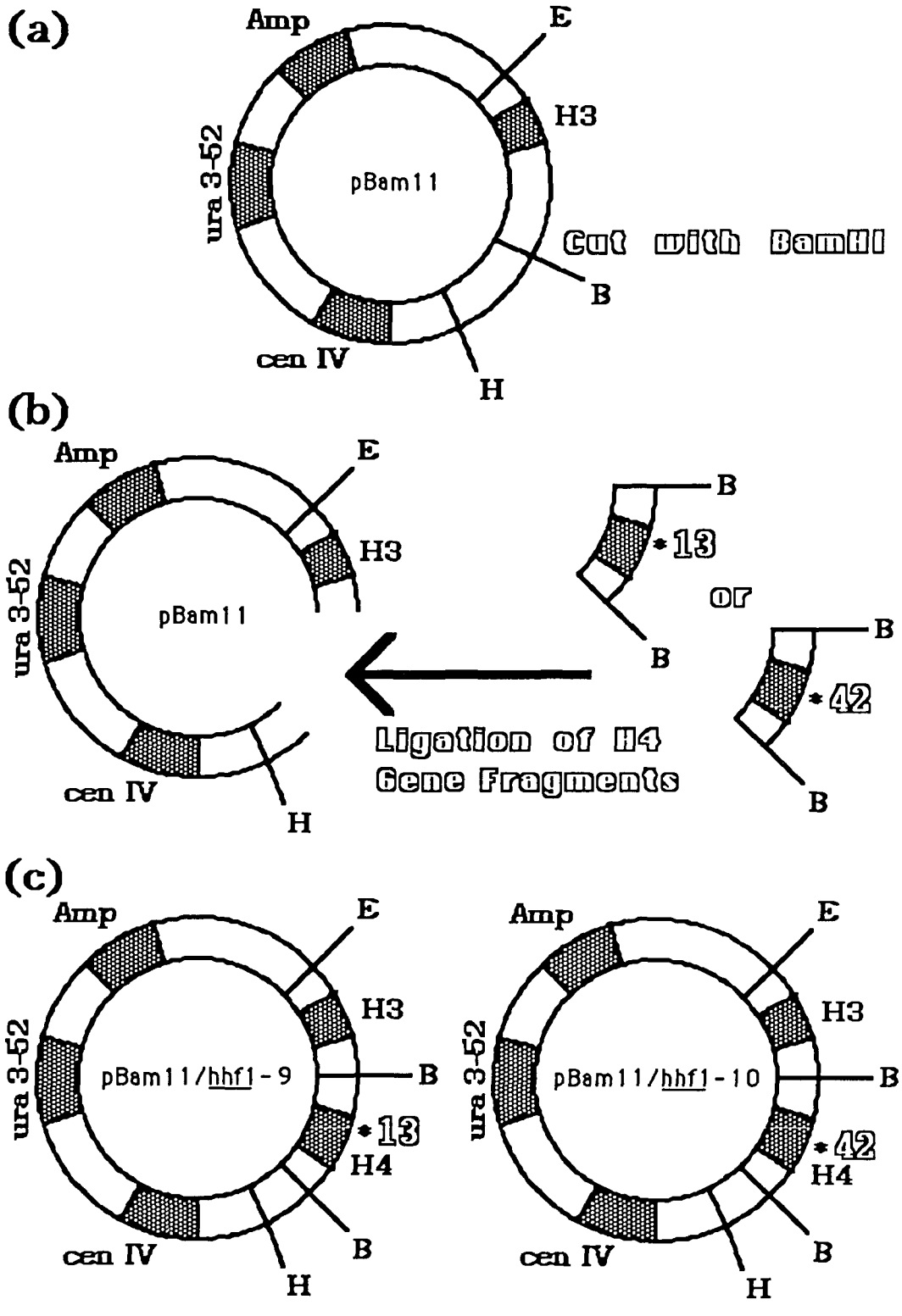
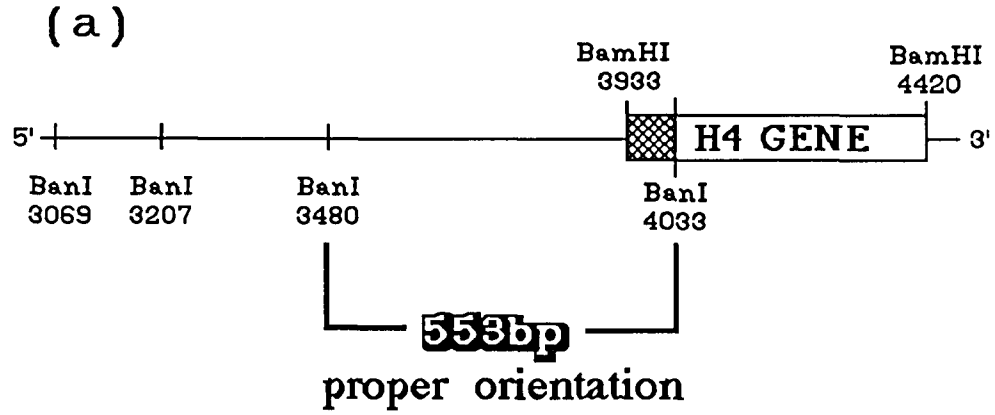

(a)
Amp
E
H3
ura 3-52
pBam11
Cut with BamHI
B
cen IV
H
(b)
Amp
E
H3
ura 3-52
pBam11
cen IV
H
B
*13
B
or
B
*42
B
Ligation of H4
Gene Fragments
(c)
Amp
E
H3
ura 3-52
pBam11/hhf1-9
B
*13
H4
cen IV
B
H
Amp
E
H3
ura 3-52
pBam11/hhf1-10
B
*42
H4
cen IV
B
H

Figure 4. H4 Orientation

A 533bp DNA band (a) would confirm proper orientation of the H4 gene. However, if the H4 gene was inverted (b) the H4-BanI site would be at position 4320 yielding a 840bp DNA band upon electrophoresis. BanI sites are numbered down stream from the centromere.


(a)
BamHI
3933
BamHI
4420
5'
H4 GENE
3'
BanI
3069
BanI
3207
BanI
3480
BanI
4033
553bp
proper orientation

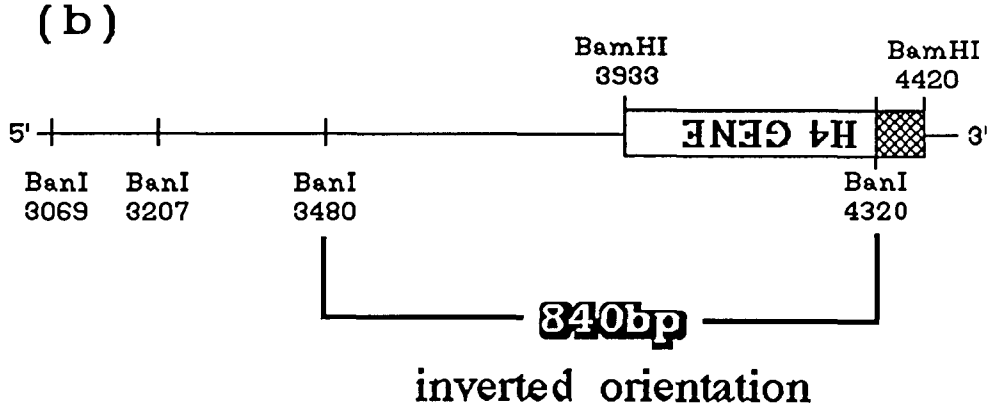

(b)
BamHI
3933
BamHI
4420
5'
H4 GENE
3'
BanI
3069
BanI
3207
BanI
3480
BanI
4320
840bp
inverted orientation

Figure 5. Plasmid Shuttle System and Plasmid Dependence Test

A representation of the plasmid shuttle system and dependence test is illustrated. The Shuttle system is designed to shuttle plasmids in and out of the yeast cell. The plasmid dependence test allows for an analysis of an H4 mutation. (a) An H4 mutation is transformed into a yeast cell deleted for both chromosomal gene sets of H4 (Δ*HHF1* and Δ*HHF2*) and surviving solely on a plasmid borne copy of the H4 gene. Transformed yeast cells are grown on selective media. Double-plasmid cells (b) are grown in selective media in an attempt to "shuttle out" the wild type H4 gene (c), provided the cell can survive with the mutant gene.

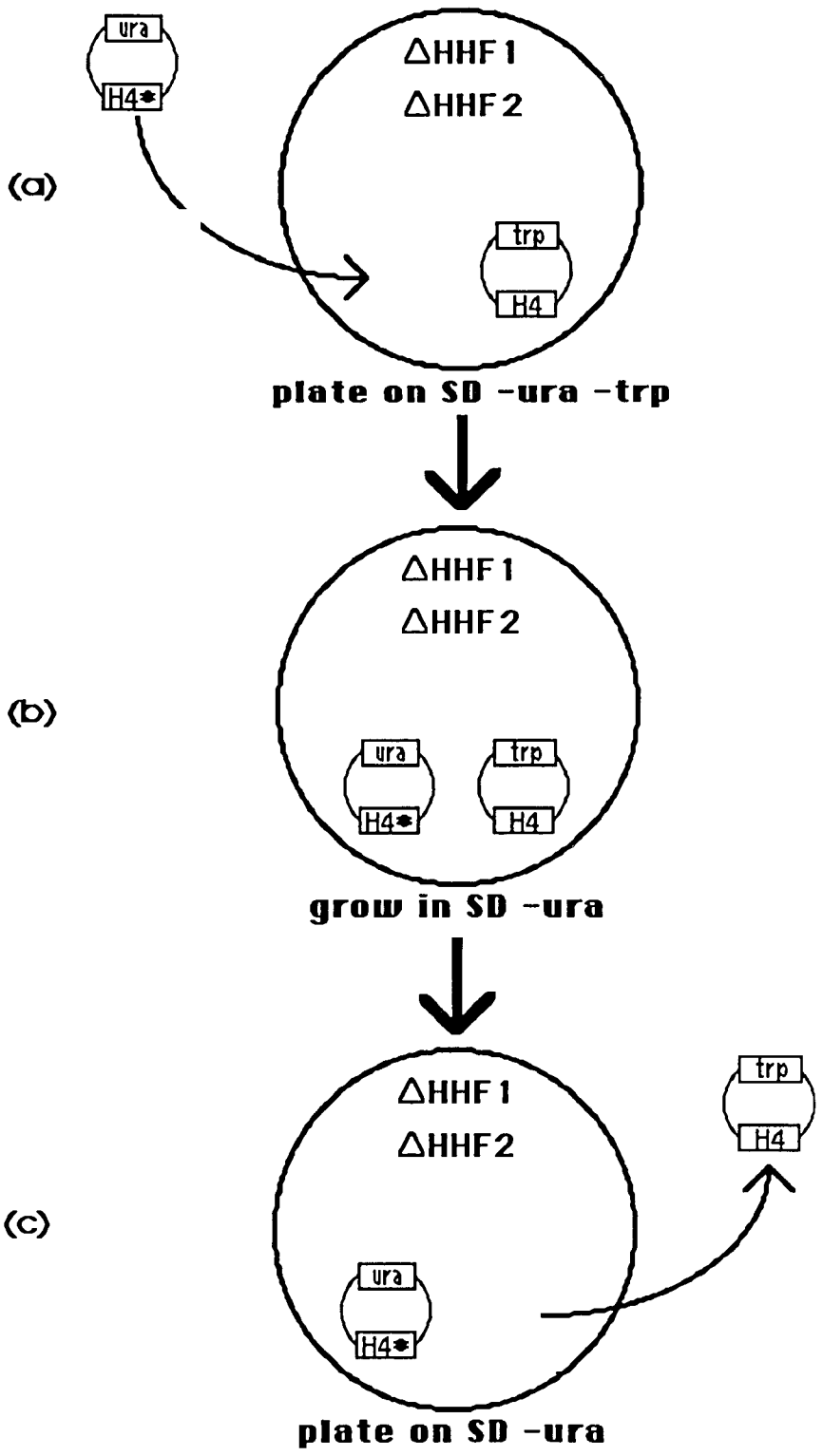

(a)
ura
H4
ΔHHF1
ΔHHF2
trp
H4
plate on SD -ura -trp
(b)
ΔHHF1
ΔHHF2
ura
H4
trp
H4
grow in SD -ura
(c)
ΔHHF1
ΔHHF2
ura
H4
trp
H4
plate on SD -ura

Figure 6. Histone H4 Protein Alignments

Consensus sequence was generated for the histone subgroup H4 and numbering is based on the consensus sequence. A dash (-) indicates unsequenced or ambiguous regions compared to the consensus. Blank spaces in the protein alignments indicate deletions with respect to the consensus. An asterisk (*) in the consensus indicates the absence of a consensus amino acid for that position. A blank space in the consensus indicates an insertion in one or more of the protein sequences at that point. A dot (.) below a consensus amino acid indicates identity with that amino acid. The one-letter code for amino acids is: A-Ala, C-Cys, D-Asp, E-Glu, F-Phe, G-Gly, H-His, I-Ile, K-Lys, L-Leu, M-Met, N-Asn, P-Pro, Q-Gln, R-Arg, S-Ser, T-Thr, V-Val, T-Tyr. Histone H4 sequence list compiled by Dan E. Wells (Wells, 1986).

87. Chicken H4
88. Drosophila H4
89. Mouse H4
90. Newt H4
91. Sea urchin H4-Pm
92. Sea urchin H4-Sp
93. Xenopus H4-B
94. Xenopus H4-L
95. Xenopus H4-LB
96. Human H4-A1
97. Sea Urchin-Lp19
98. Sea Urchin-Lp21
99. Wheat H4
100. Neurospora H4
101. Yeast H4-Sc
102. Human H4
103. Yeast
104. Xenopus H4
105. Tetrahymena H4
106. Xenopus H4-Z
107. Trout H4
108. Pig Thymus H4
109. Bovine hepatoma H4
110. Pea seedling H4
111. Rat chloroleukemic tumor H4

Histone H4 Protein Alignments