Characterization of the Possible Role of Histone H4 in DNA Methylation

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Biology

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*Scientific language in this document is held to a minimum, necessary vocabulary is documented in appendix I.
*For differentiation purposes of this paper, names of genes are italicized while names of proteins are not.

Abstract

One of several mechanisms by which genomic expression is regulated, DNA methylation is especially important for its role in post-transcriptional gene silencing. A number of recent investigations indicate the possibility that the core histones, around which DNA is packaged into the nucleosome, play some role in the methylation of chromosomal DNA. This study will indicate to what extent histone H4 and its associated residues are interrelated with the process of DNA methylation. Such correlations will be identified by substituting each of twenty-one isolated modifications of the gene encoding histone H4 for that same gene in a suitable strain of *Neurospora Crassa*. Using methylated plasmid probes in a southern analysis of each modified strain, the presence of methylated DNA

in those strains can be observed qualitatively. A southern analysis also indicates whether, and to what extent, the various residues of histone H4 are involved in this genomic process.

Introduction

DNA, the genetic code for all living organisms, is specially organized as nucleosomal chromatin in eukaryotic cells. Genes located on that DNA are to a large extent dependent upon the conformation of that structure and upon the numerous post-translational modifications to which that structure is constantly exposed (Turner 2001). Thus chromatin organization is important to determine gene expression or silencing.

The primary unit of chromatin is the nucleosome. Each nucleosome is made up of a strand of DNA, 146 base pairs in length, wrapped around a structure of protein subunits referred to as histones (Turner 2001). Together these eight core histones—two copies of each of hH2A, hH2B, hH3 and hH4—comprise what is called the histone octet (Figure 1). The majority of each histone functions as a simple packaging unit for DNA, but the n-terminal tails of each core histone , because of their positions, are susceptible to a number of post-translational modifications including methylation, acetylation, phosphorylation, and ubiquiation. Those post-translational modifications have significant impacts on gene expression (Ringo 2004). The most striking evidence of this is the gene *dim-5* (*d*efective *i*n *m*ethylation of DNA) that, instead of encoding an expected DNA methyltransferase, codes for a histone methyltransferase.

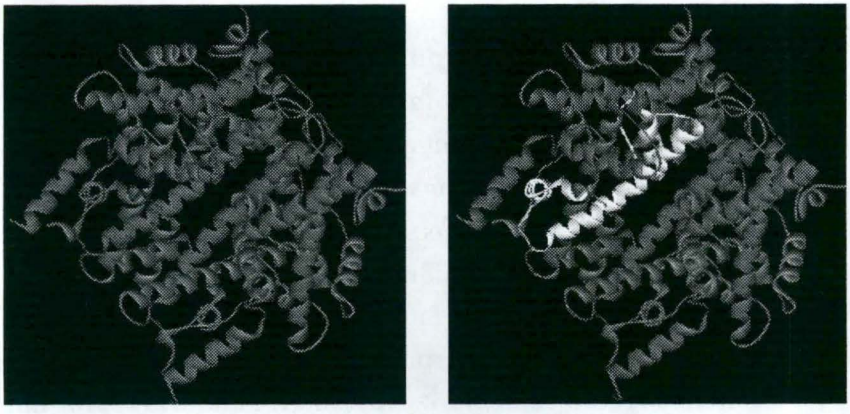


Figure 1: Crystal structure of the histone octet (left) with histone H4 highlighted in white (right).

Recent investigations that sought to characterize methylation and acetylation on specific residues of the histone H4 n-terminal tail have shown an ability of these modifications to affect gene expression. Furthermore, UO PhD graduate Shan Hayes has shown that modifications to some of the residues of the H4 tail can cause lethality and growth deficiency when using *neurospora crassa* as a model organism. The present study shows how the n-terminal tail of histone H4 can be altered so that it affects the methylation of any chromatin bound DNA.

DNA methylation, the addition of a methyl group to a cytocine base at various CpG sites, plays a significant role in the long term regulation of genomic data. DNA methylation promotes gene expression, but more often it is associated with the silencing of many unnecessary genes found in most organisms (Honda 1975). Also, methylation is a useful mechanism for specificity of cell types as genes in some specific cells are turned off while the same genes

are left on in other cells.

To study DNA methylation and its relationship to histone H4 and be applicable to numerous organisms, this study utilizes the fungus *neurospora crassa*, a model eukaryote as detailed in Roland Davis's *Neurospora: Contributions of a Model Organism.* The simplistic genomic structure of *Neurospora* allows for a reasonable level of control in gene manipulation. Also, a great number of proven experimental techniques, many developed and perfected in the Selker lab, exist for testing purposes in the organism.

This study sought to characterize histone H4 and its role (or the absence of a role) in the process of DNA methylation and therefore genomic control in the organism *Neurospora crassa.* To do this, an optimally testable strain of the organism was constructed by crossing a series of strains in the Selker Lab collection and screening for the correct genotype. Because *neurospora* has two genes encoding histone H4, the strains tested in this investigation had to contain a mutation which silences the first *hH4-1* gene such that the results of the experiments could be exclusively applicable to the precise gene to be modified *hH4-2.* Additional mutations in the genome as depicted in figure 3 (materials and methods section) are desirable for accurate southern analysis.

In Figure 3, the *hH4-1 RIP1* mutation is the test mutation with silencing in the gene encoding the first H4 histone. RIP mutations (*R*epeat *I*nduced *P*oint mutations) are base pair replacement modifications which occur specifically in methylated regions of the genome and function to silence associated genes.

The first two preliminary crosses (XFM-1 and XFM-2) provide the strains necessary to conduct a third cross, the result of which is the first desired strain, XFM-3. The products of each cross are

identified by spot testing and PCR reaction digests of DNA gathered from individual *neurospora* colonies.

The other needed strain is obtained through two more crosses (XFM-4 and XFM-5). The correct products of XFM-3 and XFM-5 are then tested by replacing the gene encoding histone H4 with a modification to that gene affecting one or more residues of the n-terminal tail of the histone protein.

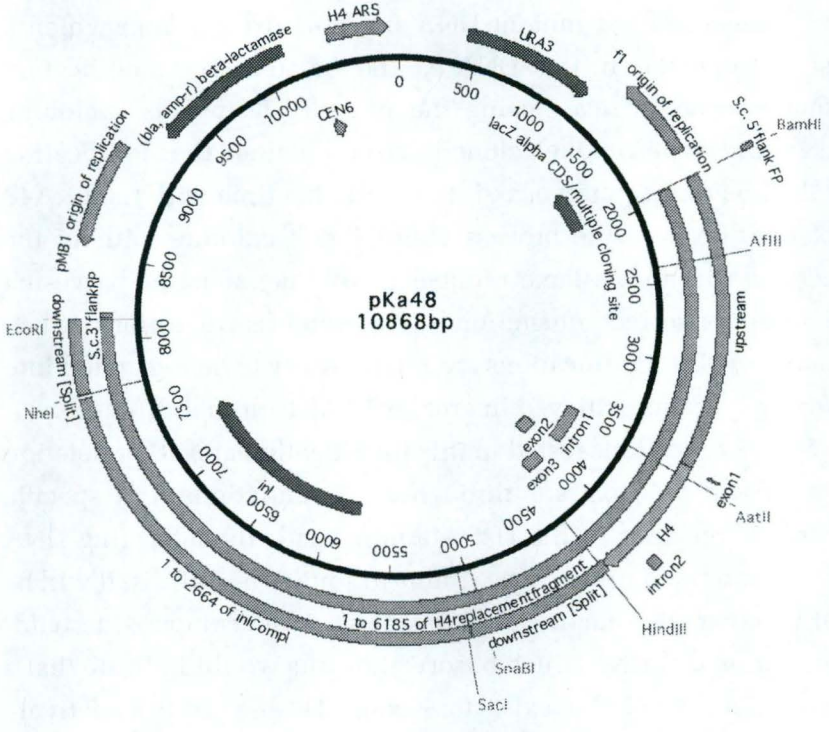


Figure 2: The pKa48 plasmid with upstream and downstream regions. Sites where restriction endonucleases used in this experiment cut the DNA are marked.

These modifications were obtained from laboratory stocks of mutant DNA and converted using the plasmid pKA48 into a usable form (see figure 2). This plasmid acts as a host for the mutant DNA allowing the transfer of H4 modifications back and forth between *neurospora* and *E-coli* cells. Also, the plasmid provides a mechanism for testing correct integration of the mutant DNA. The primary function of the pKA48 plasmid, however, is as a device to clone the mutant DNA in yeast by mitotic cell growth.

The process of preparing the H4 modifications involves first a direct transfer of the mutant DNA into E-coli cells from which a reasonable portion of that DNA can be isolated. The modification is then cloned in yeast using the pKA48 plasmid as a cloning background. When enough cloned DNA is obtained the modification can then be reintegrated back into E-coli, this time with the pKA48 backbone. Because the process yields E-coli colonies without the integrated plasmid, a large number of colonies must be harvested and tested for correct integration by endonuclease digestions. When isolated, the H4 modifications are finally ready to be integrated into the correct strains achieved in crosses XFM-1 through XFM-5.

The modifications tested in this investigation are either deletion mutations of base pairs 1 through 34 or mutations to a specific residue or residues of the H4 n-terminal tail. By subjecting DNA from the modified *neurospora* strains to southern analysis, it will be clear whether DNA methylation decreases when compared to wild-type Neurospora DNA. Such observed results would indicate that a role in DNA methylation exists for Histone H4 and create a relatively clear picture of which specific residues are actually involved.

One possible result of this study would be to open the initial idea to further investigation. The tests undertaken as part of this

research may show the viability of the Neurospora strains with these modifications at different stages of development. Some modifications of neurospora are lethal, and it is likely that others will also be lethal. Such findings would indicate not only a role in DNA methylation but furthermore a role in the methylation of functionally important sections of the genome. Also, I plan to characterize the ability of each mating type *a* or *A* to be fertile and provide further insight regarding areas of the genome that are effected by alterations in the H4 histone. Furthermore, a growth test will show whether the DNA methylation affected by the H4 n-terminal tail has any relation to growth factors.

Materials and Methods

Enzymes and Chemicals: Southern analysis probes were taken from Selker Lab stocks. All restriction endonucleases were purchased from New England Biolabs. Mus-52, am, and hH4-1 PCR primers were ordered through the University of Oregon. All other chemicals used in these experiments were ordered through the University of Oregon Lab Kitchens. All DNA used in this experiment was diluted in TE buffer and stored at -20 degrees Celsius.

Organisms and Strains: All *neurospora* strains were grown from laboratory stocks in the Selker Lab. E-coli cells containing necessary mutations for productive transformations were grown by Tamir Khlafallah in the Selker Lab. Yeast cells for cloning were borrowed from stocks in the Prehoda Lab, University of Oregon.

Crosses: All crosses were plated on 1.5% agar, SC sucrose medium containing necessary nutritional supplements (ie. histodine, inositol, alanine). The little-a mating type was allowed to grow on the medium for three days before addition of the big-A

strain. After seven more days each crossing plate was checked to assure that the paraticia (sexual structure of *neurospora*) had shot spores. In another seven days these ascospores were harvested using sterile water and stored at -20 degrees Celsius.

XFM-2:	**his-3 mat A ; hH4-1RIP1 ; am Δ inl X mat a ; am RIP8** ***mat A his-3; hH4-1RIP1 ; am RIP8***
XFM-3:	**sad-1 mat a his-3 ; mus-52Δ ::bar+; inl Δ X mat A his-3; hH4-1RIP1 ; am RIP8** ***sad-1 mat a his-3; hH4-1RIP1 ; mus-52Δ ::bar+ ; am RIP8 inl Δ*** ***mat A his-3; hH4-1RIP1 ; mus-52Δ ::bar+ ; am RIP8 inl Δ***
XFM-4:	**Sad-1 his-3 Δinl mus-52Δ::bar+ a (XFM-1)** **X** **hH4-1RIP1 Δam am-hph::am A (EB101 stock)** ***Sad-1 his-3 Δinl mus-52Δ::bar+ hH4-1RIP1 Δam am::hph::am a***
XFM-5:	**Sad-1 his-3 Δinl mus-52Δ::bar+ hH4-1RIP1 Δam am::hph::am a** **X** **Δinl Δam am::hph::am his-3::barM A (Zack Lewis' Strain)** ***Sad-1 his-3 Δinl mus-52Δ::bar+ hH4-1RIP1 Δam am::hph::am a*** ***and*** ***Sad-1 A his-3::barM Δinl Δam am::hph::am hH4-1RIP1 mus-52Δ::bar+ A***

Figure 3: Crosses XFM-1 through XFM-5. Products of each cross are highlighted in italics.

A dilution of these ascospores at 100uL spores to 500uL sterile water for each cross was heat shocked at 60 degrees Celsius for 1 hour and then plated out on plates containing a 1.5% agar, Vogel's FGS medium with necessary nutritional supplements. The next day *neurospora* colonies were harvested from these plates in amounts

depicted by Table 1 and were grown up in 1mL slants. To test whether each isolated colony possessed the desire genotype (figure 3), a number of methods were used.

Table 1: Colonies harvested from each individual cross.

	Colonies Harvested	Number of Expected Correct Genotypes	Actual Correct Genotypes
XFM-1	100	12	0
XFM-2	100	12	5
XFM-3	500	15	?
XFM-4	1000	≈ 8	?
XFM-5	200	12	?

Spot testing on liquid medium including or excluding necessary supplements identified deletion mutations. Analysis of PCR endonuclease digestions using the corresponding primers were used to identify all other genes. At this point, incorrect strains were discarded.

hH4-2 modification preparation: Stocks of mutant DNA in the Selker lab were transformed by standard procedures into *E-coli* cells (TFM-7). All modifications are listed in table 2. The *E-coli* cells were then grown for one day at 37 degrees Celsius on LB + ampicillan medium before being harvested. The DNA was isolated from these colonies using standard DNA miniprep procedures and ethanol precipitation.

Modification	Identification	Type
Δ1-15	2198	Deletion
Δ1-28	2199	Deletion
Δ1-34	2200	Deletion
Δ1-10	2201	Deletion
Δ11-28	2204	Deletion
Δ11-34	2205	Deletion
K12,16R	2206	Substitution
K5,8R	2207	Substitution
K5,8,12,16R	2208	Substitution
K5,8Q	2209	Substitution
K5,8,12,16Q	2211	Substitution
K5,8N	2212	Substitution
K5,8,12,16N	2213	Substitution
K8,12N	A	Substitution
K5,8,12N	B	Substitution
K5,8,16N	C	Substitution
K5,12,16N	D	Substitution
K8,12,16N	E	Substitution
R3A	F	Substitution
T1 A	G	Substitution
K20L	H	Substitution

Table 2: Modifications to the gene encoding histone H4's n-terminal tail

A usable section of each coding mutation was then obtained by cutting the DNA of each mutation with AflII and SnaBI. The digest of this DNA was then run out on a 0.8% agarose TAE gel (with ethyl bromide for staining) and the small band containing the desired region was cut out and eluted by standard DNA gel extraction procedures.

The plasmid pKa48 was prepared by cutting it with AatII and HindIII. The digest was then run out on a 0.8% agarose TAE gel (with ethyl bromide). The large band was then cut out and eluted.

The next step, cloning the mutation in yeast, involved combining the DNA fragments from pKa48 and the desired mutation and transforming them into yeast (TFM-8). Salmon sperm DNA was used as a component of the standard transformation solution to increase the overall efficiency. The complete transformation solution was plated on SD (–Ura) medium and left to grow for four days at 30 degrees Celsius.

After four days the yeast colonies had grown and were harvested using 2 mL of YEPD broth. A smash and grab lysis was preformed on the yeast cell suspension, and DNA was obtained for each of the mutations with a pKa48 backbone.

It was then necessary to transform the mutant DNA back into *E-coli* (TFM-9). The transformation was conducted under standard procedures and the *E-coli* cells were grown for one day at 37 degrees Celsius on LB + ampicillan medium. Twenty colonies from each transformation were harvested and grown for one more day on 2 mL liquid LB + ampicillan medium at 37 degrees Celsius. Plasmid isolations were then conducted using standard procedures to isolate the plasmid DNA from the *E-coli* cells.

To verify that any of the DNA isolated from these *E-coli* colonies

contained the correct modification, the plasmid DNA was from each colony was tested in two digests, the first being a BamHI/EcoRI digestion and the second being a HindIII/EcoRV digestion. After these were run out on a 1.2% agarose TBE gel (with ethyl bromide), a pattern consistent with the same digestion on pKa48 was observed in the correct DNA. This DNA was isolated for all mutations and used in the testing of hH4 involvement in DNA methylation.

**Testing of hH4 involvement in DNA methylation*: The DNA from each H4 modification will be transformed into each of the *neurospora* test strains (XFM-3, XFM-4). This transformation will be carried out by standard procedures and will be plated out on Vogel's FGS medium with the necessary supplements and grown for four days at 32 degrees Celsius. Colonies harvested from these plates will be grown up in 1 mL slants of the same medium for three days. Each culture will then be inoculated in liquid medium of the same composition and grown for four days. A DNA miniprep will then be conducted on these liquid grown *neurospora* cells. That isolated DNA will be run out on a 1.2% agarose TBE gel (with ethyl bromide) and a southern blot will be conducted on the gel. The blot will then be probed with methylated plasmid probes to test for any effect on methylation by any of the hH4 mutations.

**testing of hH4 involvement in DNA methylation is incomplete. See the results and discussion section*

Results and Discussion

Although the current results of this study fail to answer the original question of the H4 histone's effect on DNA methylation, the results provide some insight as to how this study can be continued and the ultimate question solved.

The bacteria *E-coli* is useful in transfer of plasmid DNA, such as the histone coding modifications used in this study, but because most viruses and bacteriophages utilize plasmid DNA, it is essential that wild type *E-coli* cell have mechanisms for combating foreign DNA. Therefore, there are restriction enzymes (much like the restriction endonuclease enzymes used in this study) present in the *E-coli* cells which cut foreign DNA into unrecognizable and hence unusable pieces (Irvine 2002). Over the years, researchers have been able to get around this problematic situation by constructing a strain of *E-coli* cells in which such enzymes are not present (Gregory 2001). Unfortunately and accidentally, the cells used in the second *E-coli* transformation (TFM-9) had been constructed incorrectly. The result was that modifications were cut beyond repair in that second transformation. This part of the experiment is easily repeatable but takes a great deal of extra time to complete again.

Unfortunately, the other half of the experimentations provided problems as well. As shown in Figure 3, the goal of several crosses was to obtain a strain which would be little a mating type, and therefore Sad-1, but also contain the mus::52-bar+ mutation. Previous studies, however, have shown the mus::52-bar+ mutation to be sex-linked, a trait overlooked in the planning of this experiment. The linkage to the y-chromosome and thus the A mating type of this mutation is not strict (not 100% in all cells) but is significant enough to drastically reduce the hope of obtaining such a strain without picking more than 1000 colonies for the second cross and many more for the third, fourth, and fifth. Recent research has indicated this sex linkage in neurospora crassa since the mutation's conception several years ago, and this study further supports such results. This finding is important in combination

with other research involving the mus::52-bar+ mutation but is nothing new and is unimportant in increasing the knowledge of gene regulation. The solution to this situation is either to pick and grow an almost unworkable number of colonies testing each for the correct genotype, or to take strains of the opposite mating type and test for the Sad-1 mutation in big A mating types. The route to be taken will be worked out for future studying of this topic.

Supplemental Materials

Appendix I: Glossary

Ascospores: The result of sexual reproduction in neurospora. These black spores are microscopic and shoot from fully matured pariticia.

Base-pair: The sequence in DNA is made up of base pairs of the nucleotides adenine, thymine,

guanine, and cytosine. (A pairs with T) and (C with G). Numbers of pairings are useful measurements of DNA length.

Chromatin: The structure of DNA and Proteins (primarily histones) housed in the nuclei of Eukaryotic cells.

Core histone: Any of the four histones (H2A, H2B, H3, H4) which comprise the histone octet.

CpG site: Site along the DNA strand where a guanine follows a cytosine base. DNA methylation occurs primarily at these sites.

C-terminal tail: hydrophobic region at the terminal end of any protein.

DIM mutation: Any genetic mutation causing a defect in the global DNA methylation process.

DNA: Deoxyribonucleic acid. The physical structure of the gene code found in the nucleus.

DNA Methylation: The addition of a methyl group to a cytosine base.

Eukaryote: Cells more complex than those of bacteria and archaea containing numerous organelles and a membrane bound nucleus.

E-coli: A bacteria widely used in research for its simplistic genomic structure.

Gene: The units of heredity in living organisms.

Gene silencing: The process on "turning off" the expression of specific genes.

Growth factors: Any protein capable of facilitating some aspect of cell division.

Histone: Any of a family of proteins found in the nucleus of eukaryotes which function in the DNA packaging of chromatin.

Histone acetylation: Addition of an acetyl group to a residue on the histone n-terminal tail.

Histone methylation: Addition of a methyl group to a residue on the histone n-terminal tail.

Histone octet: The structure of core histones around which DNA is wrapped in the nucleosome.

Histone phosphorylation: Addition of a phosphate group to a residue on the histone n-terminal tail.

in-vitro: Experimentation in a controlled environment outside the organism.

in-vivo: Experimentation of phenomena occurring inside the organism.

Mating type: The designation of male or female, or the equivalent a or A, as gender.

Methyltransferase: An enzyme (protein) which is responsible for the addition of a methyl group to a competent region of another molecule.

Meiosis: Sexual reproduction of cells involving the fusing to nuclei of opposite mating types.

Mitosis: Asexual reproduction of cells involving a direct clone by cellular division.

Neurospora Crassa: A fungal eukaryote often used in research for its simplistic genomic structure and useful applicability to higher organisms.

Nucleosome: The structure of 146 base pairs wrapped around an octet of histone proteins. This is the fundamental unit of chromatin.

N-terminal tail: The hydrophilic structure at ending with the n-terminus on a protein.

Paraticia: The black bulbous sexual structures of neurospora crassa. It is semi-visible to the naked eye. Ascospores shoot from the tube-like "beaks" at the top of each pear shaped structure.

PCR: Polymerase chain reaction. Using specific primers to amplify genes a large amount of desired DNA can be obtained in a relatively short time.

Plasmid: A circular strand of DNA, often found in bacteria, which does not code for any vital proteins in the organism.

Residue: The basic subunit of proteins. Proteins can be composed of thousands of residues bonded together in a long chain.

Restriction endonuclease: Any of a family of enzymes capable of cutting DNA at specific sites in the genome.

RIP mutation: Repeat induced point mutations are mutations of methylated DNA in which

Nonsense substitutions of base pairs are made by DNA repair mechanisms. Generally causes deactivation of genes.

Southern Blotting: The process of running DNA out on an agarose gel before transferring it to membrane which can be probed by various radioactive isotopes for specific traits of the DNA to be analyzed.

Transcription: The process by which RNA is constructed using a DNA coding template.

Translation: The process by which proteins are constructed from the RNA code.

Viability: Ability of an organism to live under different environmental circumstances.

Wild type: An organism possessing the dominant gene of a given set of alleles.

Yeast: An organism useful in research for its ability to clone plasmid DNA.

Appendix II: Raw Data and Abstract Findings

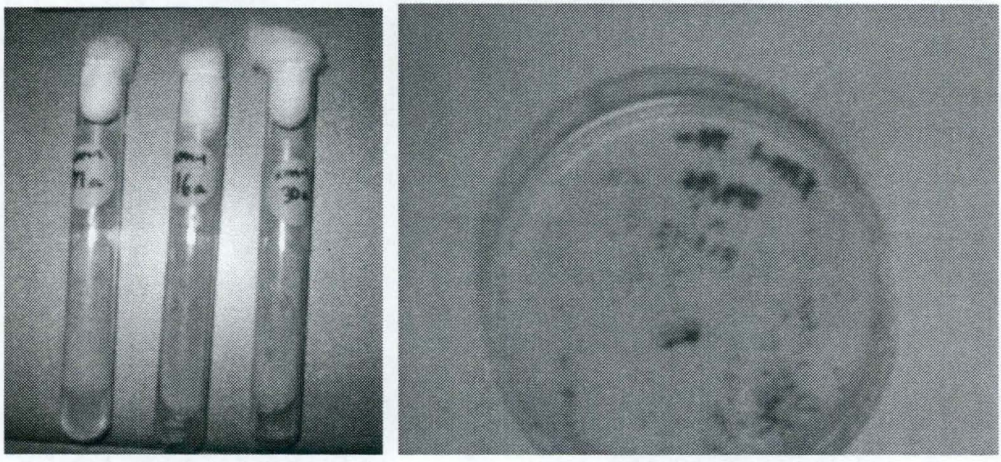


Figure 4: Parititia Growing on Crossing Medium From XFM-1 (Right) and Correct Strains Grown From Specific Colonies of XFM-1 (Left).

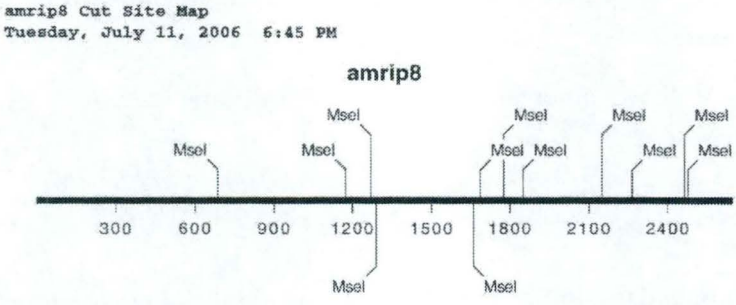
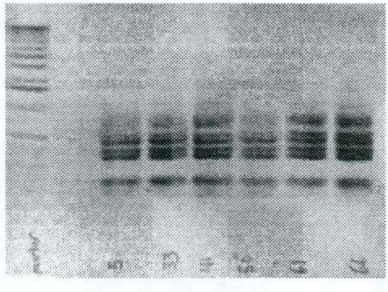


Figure 5: To test PCR products for the correct genotype, DNA from crosses, was cut with MseI which has a great number of cutting sites in the amrip8 region. In a genotype where the amrip8 gene is not present only one band of DNA would be observed on a gel.



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